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UNIVERSITY OF SUSSEX

Submitted for the Degree of Doctor of Philosophy

VICTORIA CATHERINE NORMAN

Caste and Task Allocation in Ants

SUMMARY

Group living is a widely adopted strategy by many organisms and given the advantages offered by a social lifestyle, such as increased protection from predators or increased ability for resource exploitation, a wide variety of animals have adopted a social lifestyle. Arguably none have done this more successfully than the social insects. Indeed their efficient division of labour is often cited as a key attribute for the remarkable ecological and evolutionary success of these societies. Within the social insects the most obvious division of labour is reproductive, in which one or a few individuals monopolise reproduction while the majority of essentially sterile workers carry out the remaining tasks essential for colony survival. In almost all social insects, in particular ants, the age of a worker will predispose it to certain tasks, and in some social insects the workers vary in size such that task is associated with worker morphology. In this thesis I explore the proximate and ultimate causes of worker and reproductive division of labour in ant societies, which span a range of social complexities. I predominantly focus on both the highly derived leaf-cutting ants – a so-called ‘pinnacle’ of evolution within the social insects, with a complex division of labour and a strong worker caste system – and in the more basal primitive societies of the queenless ponerine dinosaur ants, which can offer an insight in to the evolution of division of labour at the earliest stages of social lifestyles. This work demonstrates the environmental and genetic determinants of division of labour in group-living societies outside of the classical honey bee model system. This is important as it helps us to better understand the broader processes shaping behaviour and phenotype in the animal kingdom.



Caste and Task Allocation in Ants

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Submitted for the Degree of Doctor of Philosophy
University of Sussex
Faculty of Life Science
July 2016

Declaration

I confirm that the work submitted is my own, except where work which has been formed part of jointly-authored publications is included. The contributions of other authors are indicated for published chapters listed below. This thesis has not been and will not be submitted to another institution for the award of any other degree.

List of published chapters and contributions:

Chapter 2. Norman, V. & Hughes, W.O.H. 2016. **Behavioural effects of juvenile hormone and their influence on division of labour in leaf-cutting ant societies.** *Journal of Experimental Biology*. **219**: 8-11.

VN and WOHH designed the experiments, VN carried out the experiments and analysed the data and wrote the manuscript. WOHH aided in the manuscript writing.

Chapter 3. Norman, V., Pamminger, T. & Hughes, W.O.H. 2016. **The ambiguous role of juvenile hormone in regulating reproductive physiology but not dominance hierarchy in *Dinoponera quadriceps*.** *Behavioural Ecology and Sociobiology (in review)*

VN, TP and WOHH designed the experiments, VN carried out the experimental work, TP carried out dissections, VN and TP analysed the data and wrote the manuscript. WOHH aided in the manuscript writing.

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VN and WOHH designed the experiments. VN carried out the experimental work, analysed the data and wrote the manuscript. TB and FD provided technical assistance for chemical work. KT carried out preliminary behavioural trials. WOHH aided in the manuscript writing.

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VN and WOHH designed the experiments. TP carried out immunity assays. VN carried out all other experimental work, analysed the data and wrote the manuscript. WOHH aided in the manuscript writing.

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WOHH, VN, HD and SA conceived and planned the study. VN, CT and HD collected samples. VN and HD performed molecular work and analysed the data. VN and HD drafted the manuscript. All authors helped draft the final manuscript and gave approval for publication.

Chapter 8. Norman. V., Pamminger, T. & Hughes, W.O.H. 2016. **Behavioural**

development, fat reserves and their association with productivity in *Lasius flavus* founding queens. *The Science of Nature*. 103: 3-4.

VN and TP designed the experiments. VN carried out all experimental work, analysed the data and wrote the manuscript. TP and WOHH aided in the manuscript writing.

Chapter 9. Norman, V.C., Pamminger, T., F. Nascimento & Hughes, W.O.H. 2016.

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VN, TP and WOHH designed the experiments. VN carried out the experimental work, analysed the data and wrote the manuscript. FN assisted with field work. TP and WOHH aided in the manuscript writing.

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Abstract

Group living is a widely adopted strategy by many organisms and given the advantages offered by a social lifestyle, such as increased protection from predators or increased ability for resource exploitation, a wide variety of animals have adopted a social lifestyle. Arguably none have done this more successfully than the social insects. Indeed their efficient division of labour is often cited as a key attribute for the remarkable ecological and evolutionary success of these societies. Within the social insects the most obvious division of labour is reproductive, in which one or a few individuals monopolise reproduction while the majority of essentially sterile workers carry out the remaining tasks essential for colony survival. In almost all social insects, in particular ants, the age of a worker will predispose it to certain tasks, and in some social insects the workers vary in size such that task is associated with worker morphology. In this thesis I explore the proximate and ultimate causes of worker and reproductive division of labour in ant societies, which span a range of social complexities. I predominantly focus on both the highly derived leaf-cutting ants – a so-called ‘pinnacle’ of evolution within the social insects, with a complex division of labour and a strong worker caste system – and in the more basal primitive societies of the queenless ponerine dinosaur ants, which can offer an insight in to the evolution of division of labour at the earliest stages of social lifestyles. This work demonstrates the environmental and genetic determinants of division of labour in group-living societies outside of the classical honey bee model system. This is important as it helps us to better understand the broader processes shaping behaviour and phenotype in the animal kingdom.

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1 General Introduction

Many of the most ecologically successful species have evolved a social lifestyle (Krause and Ruxton 2002). Living as a group can offer many benefits, such as protection from predators, a greater ability to exploit resources and increased reproductive fitness (Krause and Ruxton 2002; Dornhaus et al. 2012). Consequently, a wide diversity of animals, from mammals to insects, have adopted a group-living lifestyle over the course of evolutionary history. A key component of the success of group-living is task allocation among individuals, such that individuals can specialise, temporarily or permanently, in one or few roles (Bourke and Franks 1995; Beshers and Fewell 2001).

1.1 Reproductive division of labour in advanced eusocial societies

The defining characteristic of division of labour in social insects is reproductive skew and this phenomenon is observed across the animal kingdom (Jamieson 1997; Cuvillier-Hot et al. 2004a; Neff et al. 2008). In communally breeding species, reproductive output is not equally distributed among the potential pool of breeding group members. How such skew is generated and the processes maintaining this are fundamental questions in biology. Eusocial insects (ants, termites, some bees, some wasps, and a few aphid, thrip and beetle species), however, have emerged as a key model system for testing many of the hypotheses surrounding reproductive skew (Reeve and Keller 2001) and provide some of the most complex examples of group-living and division of labour (Oster et al. 1978; Robinson 1992). Eusocial insects are defined by two or more overlapping generations caring cooperatively for young, as well as a split within the group (either behaviourally or physically) into reproductive and non-reproductive castes (Crespi and

Yanega 1995; Wilson and Hölldobler 2005) In many, though not all, eusocial insects the reproductive, or queen caste, is morphologically adapted for reproduction with the remaining, more-or-less sterile, workers carrying out other tasks such as food collection and brood care (Wilson 1976). This split is responsible for the huge ecological and evolutionary success of these organisms allowing the essentially sterile workers to efficiently carry out the remainder of the tasks fundamental for colony success. This has resulted in them being considered a major evolutionary transition in evolution (Wilson 1990; Smith and Szathmary 1997; Hölldobler and Wilson 2009). A unique exception to this, however, are the primitively eusocial species (see below).

1.2 Worker division of labour in advanced eusocial societies

Further to the reproductive division of labour, social insects also show division of labour among the workers. The process of self-organisation among the workforce is a remarkable feat, where, instead of hierarchical system of task allocation workers respond to local cues in order to engage in the tasks immediately required by the colony (Theraulaz et al. 1998; Beshers and Fewell 2001). Such tasks include foraging, waste removal, brood tending, and guarding behaviours (Hölldobler and Wilson 1990). How these jobs are allocated depends on multiple factors including genotype, age, experience, morphological caste, and physiology (Sanada-Morimura 2003; Jones et al. 2004; Seid and Traniello 2006; Chapman et al. 2007; Schwander et al. 2008; van Wilgenburg et al. 2010). Age-related division of labour, or temporal polyethism, is a key mechanism in allocating tasks among individuals in almost all social insect colonies, from wasps, through to highly eusocial ant and bee societies (Seeley 1982; Traniello and Rosengaus 1997; Robinson 2002; Shorter and Tibbetts 2008; Bloch et al. 2009; Torres et al. 2012). Typically younger workers perform tasks inside the nest, such

as brood care or nest maintenance, whereas older individuals carry out the more dangerous, external tasks such as foraging. This age related division of labour is seen in almost all social insect species (Table 1.1) and is a classic example of how response thresholds to certain behavioural stimuli can change over the course of an individual's lifetime (Oster et al. 1978; Robinson 1992; Jones et al. 2004; Hölldobler and Wilson 2009).

1.3 Morphological worker caste systems in advanced social societies

Some social insects have taken the division of labour a step further where the task allocation among the workforce is associated with worker morphology (Oster et al. 1978). This size-related polyethism can be non-discrete, as is seen in bumblebees and some ants, with workers exhibiting a continuous range of sizes that then influences task, with smaller individuals typically carrying out within-nest tasks and larger individuals colony defence and foraging, (Oster et al. 1978; Beshers and Fewell 2001; Goulson et al. 2002). However, in some of the largest and most complex of social insect colonies, specific worker phenotypes are often categorised into castes which predispose an individual to a task based on their physical attributes (Wilson 1976; Wetterer 1995). Workers develop into different castes due to differences in allometric growth during larval development. These caste systems are shown in the termites and approximately 15% of ant genera, as well as at least one species of stingless bee species with some ant societies showing extreme worker caste systems (Oster et al. 1978; Hölldobler and Wilson 1990; Thorne et al. 2003; Grüter et al. 2012).

In leaf-cutting ants in particular, individuals display extreme phenotypes within colonies, some individuals being orders of magnitude larger than their sisters (Hölldobler and Wilson, 2010; Wilson, 1980; Figure 1.1). The specific morphological

adaptations, such as those which specialise individuals towards foraging, or the extraordinarily large soldiers of the *Atta* genus adapted for colony defence, enable individuals to be much more efficient at the task required compared to an unspecialised individual (Wilson 1983). Individuals will, however, undertake almost any role if the stimulus is strong enough (Theraulaz et al. 1998; Beshers and Fewell 2001; Evison and Ratnieks 2007; Waddington et al. 2010). This may increase the overall efficiency of the colony and allows these ants to occupy a highly specialised niche very successfully. Such roles include, but are not limited to, tending to the fungus garden, care of larvae and pupae, the transportation and cutting of leaves, and defence against conspecifics and vertebrate predators (Hölldobler and Wilson 2010). This astonishing division of labour, both temporal, and phenotypic, makes this study system an ideal one within which to study the causes and consequences of caste and task allocation.

1.4 Does division of labour increase efficiency?

Recent work has started to question the fundamental assumption that specialised workers outperform generalists at particular tasks. This has become an area of real contention within the social insect world and is open for serious debate. Recent evidence has suggested that that individual specialisation in monomorphic *Temnothorax* ants does not correlate with efficiency at that task (Dornhaus 2008), although evidence from social spiders, which lack morphological castes but show specific behavioural phenotypes showed that aggressive behavioural phenotypes were more successful at prey capture than their docile counterparts (Wright et al. 2014) . And, indeed, the within societies with morphologically specialised castes it has been argued that the advantage for this came from having the correct numbers of each caste, supposedly specialised for its specific role (Oster et al. 1978). This would then lead to situations in

environments that favour more foraging selection act on colonies to produce caste ratios in favour of the larger castes that are specialised in such a role (Gordon 2015)

However, our evidence to date is limited and that which we do have suggests such selective pressures have not been demonstrated (Beshers and Traniello 1996). This all suggests that task allocation changes in response to shifting conditions and the needs of the colony (Gordon 1989). Here, a worker's task depends on the current availability of other workers to perform it and the number of workers involved in other tasks (Wilson 1984; Huang et al. 1998). The task an individual worker performs is not just to do with its caste (be that age or morphological caste) but the result of a shifting set of interactions both among individuals and their interaction with the environment (Gordon 2015). There are also arguably other benefits to the division of labour and subsequent production of producing specialised individuals excluding individual efficiency increases. Such benefits may include a decreased cost associated in switching between tasks, increased spatial efficiency, as hypothesised in ants, or may simplify the processes of task allocation by minimising neural, or other, costs associated with the physiological process of task allocation itself (Jeanne 1986; Sendova-Franks and FRranks 1995; Chittka et al. 1997; Dornhaus 2008).

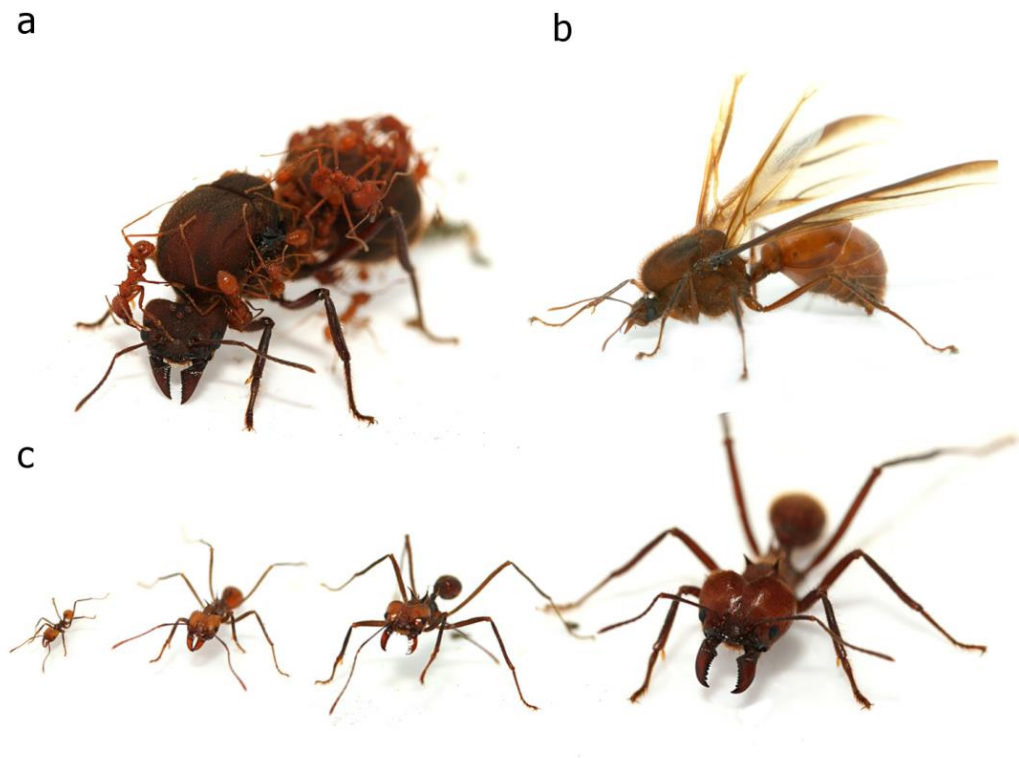


Figure 1.1 The caste system in *Atta cephalotes* leaf-cutting ants. a) A queen surrounded by attendant nurse workers, b) Winged male c) the worker caste system showing massive size differences between sister workers, including a soldier caste (far right), unique within the leaf-cutting ants to the genus *Atta*.

1.5 Primitive social organisation

Primitive species, therefore, offer a unique perspective into the evolution of unequal reproductive output and subsequent processes generating division of labour. In many species, including non-eusocial species, reproductive status is acquired by one or few individuals during antagonistic interactions that result in the establishment of dominance hierarchies. These hierarchies are found in a number of social organisms, both vertebrate and invertebrate (Monnin and Peeters 1999; Monnin et al. 2003; Graham and Herberholz 2009; Hewitt et al. 2009; Chiarati et al. 2010). Understanding the processes governing the acquisition of these hierarchies and how they are

maintained is a fundamental question for both ecology and evolution. These hierarchies are often characterized by rank-specific syndromes, both of physiological, or reproductive, traits as well as behavioural, which can clearly separate high and low ranking individuals and these hierarchies, often dictate which individuals get to reproduce (Monnin and Ratnieks 2001; Monnin et al. 2003).

Ants in particular have emerged as useful model systems for testing the proximate and fundamental reasons for these questions as they span a wide range of social complexity within their societies. While the ‘classical’ life history trait within the highly eusocial insects is that of a morphologically specialised queen caste, some genera (approximately 100 species) have secondarily lost the queen caste (Medeiros et al. 1992; Monnin and Peeters 1999; Lommelen et al. 2010; Penick et al. 2011). In these more socially ‘primitive’ societies, in which all individuals within a colony are monomorphic and all workers have the potential to reproduce, there is strong potential conflict between group members over reproduction (Peeters 1997; Monnin and Peeters 1999; Monnin and Ratnieks 2001). Within the genera that have reverted to their primitive, queen-less state, reproduction is monopolised by mated, dominant, individuals termed ‘gamergates’ (Peeters 1997). Within these gamergate groups, conflicts over reproductive rights are resolved through ritualised, aggressive behaviours which result in the formation of a dominance hierarchy in which one, or in some cases, a small group of workers are the sole individuals to contribute to female destined eggs for the colony. Of course, some individuals may cheat and lay male eggs (Monnin and Peeters 1998; Monnin and Ratnieks 2001)

One such species that has become a model system for studying such dominance hierarchies within the ants is the dinosaur ant *Dinoponera quadriceps*. This ponerine ant, from Brazil, forms relatively small colonies and shows a gamergate system in

which only one dominant worker, the alpha, mates and produces sexual offspring, thereby being the functional equivalent of a queen (Monnin and Peeters 2008). The alpha actively suppresses her subordinates through stereotyped, ritualized behaviours, thereby rendering the remaining, submissive, workers in the colony functionally sterile as well as indirectly suppressing challengers by placing pheromone on them (Monnin and Peeters 1999). These workers form a linear hierarchy after the alpha, whereby the beta, or another high ranker, may take over from the alpha if she dies (Grainger et al. 2014). High-ranked individuals therefore tend to undertake less 'risky' tasks, and are often found within the brood chamber, which increases their survival chances and therefore allows individuals to maintain their reproductive potential (Nascimento et al. 2012; Asher et al. 2013; Grainger et al. 2014). How these hierarchies are physiologically translated into behavioural specialisation is a poorly understood area of ecology and evolutionary biology.



Figure 1.2 Defensive, foraging and brood care behaviours in ants. a) An aggressive display from *Camponotus sericeiventris*, b) *Atta laevigata* soldier, c) foraging *Atta colombica*, d) dominance behaviours in *Dinoponera quadriceps*, e) foraging *Acromyrmex octospinosus*, f & g) fungus gardens and brood care behaviours in *Atta colombica*, h) brood care behaviours in *Dinoponera quadriceps*.

1.6 Factors affecting division of labour

Two main factors are thought to affect division of labour (both reproductive and within-worker) across the social insects: environmental cues and genotypic effects (Robinson 1992; Schwander et al. 2005; Smith et al. 2008b; Smith et al. 2008a). Behaviour, and caste, however are almost certainly a combination of these two factors within most social insects (Via and Lande 1985; Hughes and Boomsma 2007; Anderson 2008). The endocrinological system is of particular importance for mediating environmental cues on both reproductive, and worker division of labour.

1.6.1 The endocrinological system

1.6.1.1 Physiological mechanisms mediating reproductive division of labour

Hormones are prime candidates for the proximate mechanisms controlling both the morphological, and behavioural, castes observed across the social insects. One hormone of particular interest in insects is juvenile hormone (JH). Juvenile hormone's classic role within solitary insects is as a gonadotropin (Nijhout 1998). It paces reproductive development, with an increase in the hormone titre causing an increase in vitellogenin (an egg yolk and storage protein) synthesis in the fat body, vitellogenin uptake by developing oocytes, and then egg production (Borst et al.; Comas et al. 1999; Hartfelder 2000). JH is also important in regulating other aspects of insect reproduction, such as maternal behaviour and body size, aggression, oviposition behaviour, and flight behaviour (Barth et al. 1975; Nijhout 1998; Bloch et al. 2000; Flatt et al. 2005; Bloch et al. 2009; Helms-Cahan et al. 2011).

In contrast, the role of JH in social species is more complex. In some 'highly' eusocial taxa, such as honey bees and *Lasius* queens, JH has lost its ancestral

gonadotropic function and instead a high JH titre in reproductive individuals is associated with a decrease in reproductive ability (Engels et al. 1990; Nijhout 1998; Pinto et al. 2000; Corona et al. 2007; Pamminer et al. 2016). Interestingly, JH does, however, regulate reproductive physiology in pre-adult stages (Capella and Hartfelder 1998; Bloch et al. 2009). For example, the physiological effect of diet on queen caste determination in honey bees, in which larvae fed a diet rich in amino acids are stimulated to continue developing in to queens rather than follow a worker developmental pathway, is mediated by juvenile hormone (Haydak 1970; Rembold et al. 1974; Corona et al. 2007; Kamakura 2011). Juvenile hormone is also associated with queen determination in bumblebees (Cnaani and Borst 1997), with larvae exhibiting higher levels of juvenile hormone biosynthesis compared to worker larvae. Instead of regulating adult reproductive physiology and behaviour, evidence from the highly eusocial societies of honey bees suggests that JH has switched roles to serve as a behaviour pacemaker within the sterile workers (see next section).

Interestingly, JH has retained its ancestral, gonadotropic role in a number of more ‘primitively’ eusocial species, such as bumblebees and paper wasps (Röseler 1977; Bloch et al. 2000; Tibbetts and Huang 2010; Amsalem et al. 2014; Kelstrup et al. 2014). However, in contrast, some ‘primitively’ eusocial ant species with gamergate systems show a decrease in fertility among the dominant, reproductive individuals (Cuvillier-Hot et al. 2004b; Penick et al. 2011).

1.6.1.2 Physiological mechanisms mediating worker division of labour

There is increasing evidence across ‘higher’ or ‘advanced’ eusocial taxa that instead of a reproductive role, JH has been co-opted to act as a behavioural pacemaker for adult worker behaviour. In almost all social insects, age-polyethism is observed: the response threshold of an individual to certain tasks is flexible over the course of its lifetime.

Individuals of a young age have low thresholds for tasks based within the nest, whereas older individuals will have low thresholds for more ‘risky’, external, tasks such as foraging or nest defence (Robinson 1992). This age polyethism, at least within the model system of honey bees has been proved, experimentally, to be under the control of juvenile hormone: an increase in the titre of hormone in an individual as it ages is responsible for shifting its task thresholds for these behavioural stimuli and young bees treated with a hormone mimic accelerate their behavioural development to become precocious foragers (Sullivan et al. 2000; Schulz et al. 2002).

There is also increasing evidence across social taxa more broadly, including several ant, bee and wasp species that older, foraging workers show higher JH titres than their younger, within-nest sisters (Dolezal et al., 2012, 2009; Giray et al., 2005; Lengyel et al., 2007; Shorter and Tibbetts, 2008; Table 1.1). This alternative, behavioural pacemaker role for JH, regulating the age-related behavioural switch to foraging is thought to have evolved through co-option of the endocrine signalling pathways which would have been associated with reproductive regulation in basal, solitary ancestors (Amdam et al. 2004; Penick et al. 2011).

Juvenile hormone is also implicated in physical worker caste determination through experimental application of JH analogues during larval development (Wheeler and Nijhout 1981; Rajakumar et al. 2012). This hormone is vital to insect development in general but when applied to the larvae of *Pheidole bicarinata* during a specific window in the last larval instar it has been shown to stimulate soldier production. It has been suggested that one way in which nutrition may act as an environmental predictor of caste determination is through acting on juvenile hormone biochemical pathways once a threshold of nutritional quality and/or quantity is reached (Wheeler and Nijhout 1984).

Table 1.1 Examples of age polyethism across social insects and, where known, how juvenile hormone affects behavioural maturation

Species	Type of polyethism	Reference
Bees		
<i>Apis mellifera</i>	Workers exhibit strong age polyethism and can be induced by topical JH application	(Robinson 1987a; Giray et al. 2000)
<i>Trigona minangkabau</i> (stingless bee)	Workers exhibit no age polyethism, rather show lifetime specialisation for a task.	(Inoue et al. 1996)
<i>Bombus</i> spp. (Bumble bees)	No evidence for age polyethism and worker division of labour is not affected by JH	(Cameron 1989; Cameron and Robinson 1990)
Ants		
<i>Acromyrmex</i> spp.	Exhibits strong age polyethism across multiple species which can be accelerated by topical JH application	(Camargo et al. 2007; Waddington and Hughes 2010; Norman and Hughes 2016)
<i>Trachymyrmex septentrionalis</i>	Exhibits age polyethism (and weak size polyethism)	(Beshers and Traniello 1996)
<i>Myrmecaria eumenoides</i>	Exhibits age polyethism and external task correlated with JH titre	(Lengyel et al. 2007)
<i>Pheidole hortensis</i>	Exhibits age polyethism	(Calabi et al. 1983)
<i>Camponotus floridanus</i>	Exhibits age polyethism	(Tripet and Nonacs 2004)
<i>Pogonomyrmex</i> spp.	Exhibits age polyethism and external task correlated with JH titre	(Gordon et al. 2005; Dolezal et al. 2012)
<i>Leptothorax acervorum</i>	Exhibits age polyethism	(Kühbandner et al. 2014)
<i>Myrmica scabrinodis</i>	Evidence for age	(Moroń et al. 2008)

	polyethism – ants with shorter lifespans will adjust their behavioural maturation accordingly	
<i>Harpagathus saltator</i> (primitively eusocial)	Evidence for age polyethism and external task correlated with JH titre	(Penick et al. 2011; Haight 2012)
<i>Ectatomma tuberculatum</i> (primitively eusocial)	No strong evidence for age polyethism – old workers observed tending to brood	(Peeters 1997)
<i>Nothomyrmecia macrops</i> (primitively eusocial)	Primitively eusocial: no strong evidence for age polyethism - All colony members observed partaking in all tasks	(Jaisson et al. 1992)
<i>Prionopelta amabilis</i> (primitively eusocial)	Evidence for age polyethism – older workers more engaged in foraging	(Hölldobler and Wilson 1986)
<i>Amblyopone pallipes</i> (primitively eusocial)	Weak evidence for no age polyethism	(Traniello 1978)

Wasps

<i>Polistes canadensis</i>	Evidence for age polyethism which is accelerated by topical JH application	(Giray et al. 2005)
<i>Polistes diminulus</i>	Evidence for age polyethism which is accelerated by topical JH application	(Shorter and Tibbetts 2008)
<i>Mischocyttarus consimilis</i> (eusocial wasp)	Evidence for age polyethism	(Torres et al. 2012)
<i>Polybia occidentalis</i> (large-colony swarm founding wasp)	Age positively correlated with external activities and is accelerated by JH application	(O'Donnell and Jeanne 1993)
<i>Ropalidia marginata</i>	Evidence for age	(Naug and Gadagkar

(primitively eusocial) polyethism 1998)

Termites

<i>Zootermopsis angusticollis</i>	No evidence for age polyethism	(Rosengaus and Traniello 1993)
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1.6.2 Genetic mechanisms

1.6.2.1 Genotypic influences on worker behaviour

One mathematical model which tries to explain the division of labour in social insects is the response threshold model (Theraulaz et al. 1998). Under this model of task allocation the likelihood that any one worker will engage in a specific task depends both on the level of the stimulus for that task as well as that worker's individual threshold for that stimulus (Theraulaz et al. 1998; Beshers and Fewell 2001). For example, workers with a low threshold will undertake tasks with a relatively low stimulus, whereas those with a higher threshold for that task will require a much stronger stimulus in order to undertake that particular role (Fewell and Page 1993; Beshers and Fewell 2001). If a colony contains workers with varying response thresholds for every task, this is one way in which the workforce can self-organise and generate task specialisation and subsequent division of labour. Much of the early work on response thresholds come from the model system of honey bees. The response threshold of a worker changes over the course of her lifetime, such that the tasks performed by a worker change as she ages (Robinson 1992).

An important factor affecting an individual worker's task threshold is genotypic variation. Honey bee queens show some of the highest levels of polyandry within social insects, with queens mating with ~10-30 males on their nuptial flight. Colonies are

made of patriline – subsets of workers fathered by the same male (Boomsma and Ratnieks 1996; Hughes et al. 2008). This is often associated with significant costs to the virgin queens such as increased energy expenditure, increased predation risk and both direct and indirect evidence for negative implications of polyandry on immunity (Crozier and Fjerdingstad 2001; Sumner et al. 2004; Baer et al. 2006; Roberts et al. 2015; Helms et al. 2016).

Increased genetic diversity through polyandry has been hypothesised to benefit the colony by more efficiently dividing the labour among the workforce – with observed differences between patriline in their frequency of task performance suggesting a strong genotypic influence on varying task thresholds between patriline (Crozier and Page 1985; Theraulaz et al. 1998; Oldroyd and Fewell 2007). Evidence has been found for patriline showing predispositions for a number of behaviours ranging from hygienic behaviours, thermoregulation, preferred forage, guarding, corpse removal and age at onset of foraging (Calderone and Page 1988; Robinson and Page 1988; Robinson 1992; Fewell and Page 1993; Page et al. 1995b; Page et al. 1995a; Fewell and Page Jr 2000; Breed et al. 2004; Julian and Fewell 2004; Jones et al. 2004).

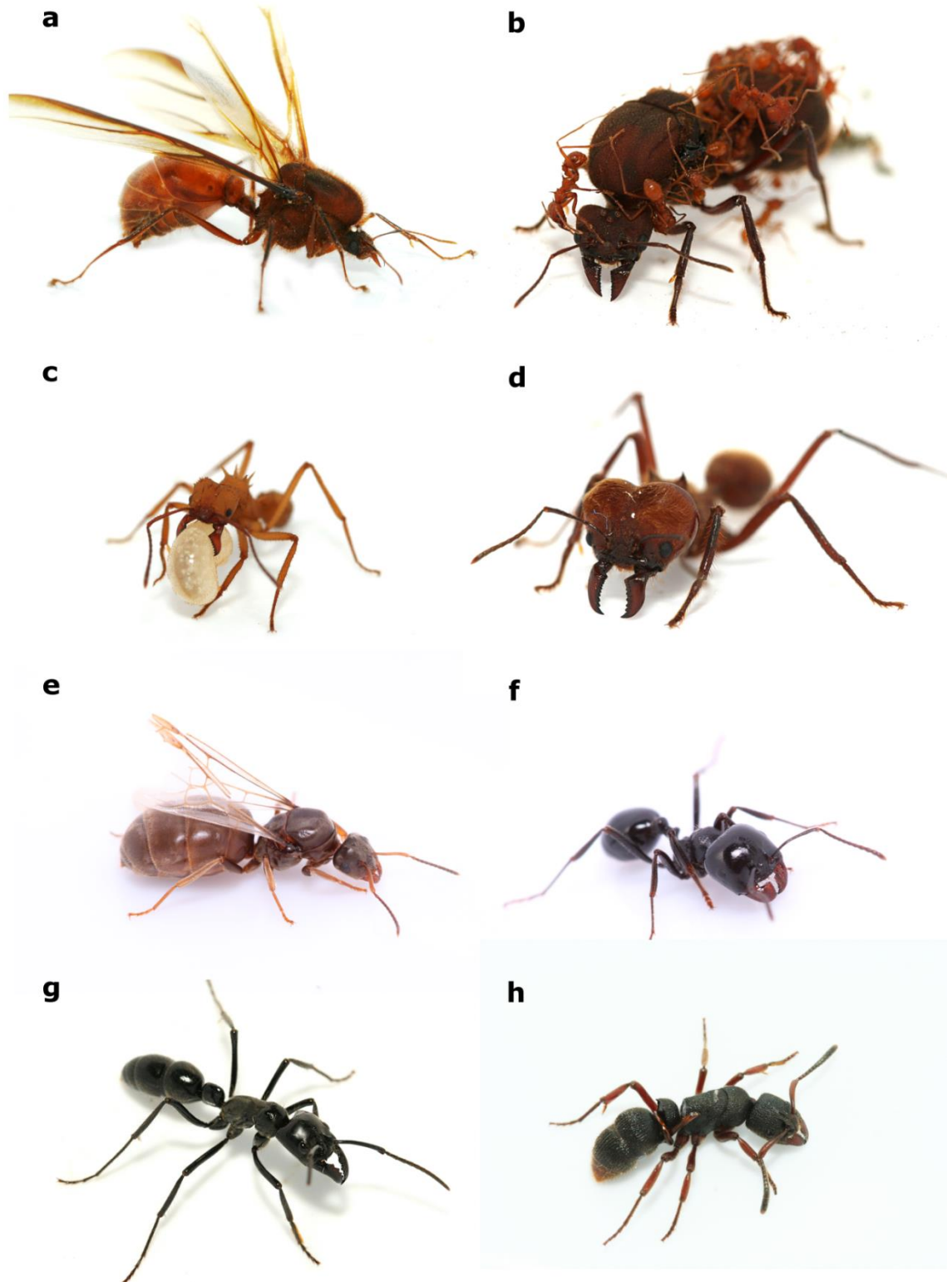


Figure 1.3 Some of the study species used in this thesis. a) *Atta cephalotes* male and b) mother queen. c) *Acromyrmex octospinosus* brood tender, d) *Atta cephalotes* soldier, e) *Lasius niger* winged virgin queen, f) *Messor barbarus* worker, g) *Dinoponera quadricaps* worker and h) *Pachycondyla rufipes* worker.

Genetic effects on polyethism have also been discovered in a number of ant species. Task preference as well as physical caste has recently been shown to have a genetic basis in the leaf-cutting ant *Acromyrmex echinator*, as well as *Formica selysi* and *Cataglyphis cursor* (Schwander et al. 2005; Fournier et al. 2008; Waddington et al. 2010; Eyer et al. 2012). Furthermore, patriline can also affect the ability with which *A. echinator* individuals carry out certain tasks (Constant et al. 2012). This highlights the complexity of how tasks are distributed within a colony, whereby in leaf cutting ant colonies tasks are not only influenced by caste but a blend of factors including genotypes, castes, age and experience.

1.6.2.2 *Genotypic influences on worker caste determination*

A number of ant species exhibiting a worker caste system have also been shown to have a genotypic element to worker caste determination as well as task propensity (Fraser et al. 2000; Hughes et al. 2003; Rheindt et al. 2005; Schwander et al. 2005; Jaffé et al. 2007; Smith et al. 2008a) (table 2). If all individuals were totipotent with respect to caste, we would expect caste allocation to be based entirely on environmental cues (Oster et al. 1978). Genotypic influences on caste could, in theory, constrain the ability of a colony to adjust caste ratios in a flexible manner, particularly so where tasks are related to physical caste. However, within these ant societies which show a genotypic predisposition for certain castes, all patrilines are often detected within all physical castes. This is suggestive that genotype only biases towards certain castes and likely interacts with environmental thresholds to produce optimal caste ratios required by the colony (Fraser et al. 2000; Hughes et al. 2003; Anderson 2008; Smith et al. 2008b; Constant et al. 2012).

For example, in the leaf-cutting ant species *Acromyrmex echinator* the representation of patrilines within a colony shifted after removal of large workers

(Hughes and Boomsma 2007). Upon increasing the stimulus for large workers a range of genotypes developed into large workers. In control colonies, which had a range of worker sizes removed, genotypes were similarly represented in large workers both before and after worker removal (Hughes and Boomsma 2007). It is thought that this genotypic variation in predisposition may allow the colony to more easily adapt to its changing needs through gene-by-environment interactions (Smith et al., 2008a). This suggests that this phenotypic plasticity, as with polyethism in honey bees, is generated by genotypes differing in their response to the environmental cues and the changing needs of the colony, although the proximate causes are not clear. How colonies and individuals react to stimuli to increase a particular caste is likely to depend on the role of that caste within the colony. It might be expected that roles that are more pressing, e.g. foraging, would be replaced sooner and with more genotypic flexibility than a caste with a less vital role.

1.6.2.3 *Hard-wired genetic caste determination*

In some cases, caste has been shown to be genetically hard-wired – with essentially no environmental input in to caste determination (Table 1.2). Such cases are, at present, comparatively rare and limited to five genera. The first example of hard-wired genetic caste determination came from *Pogonomyrmex* harvester ants and involves a remarkable mating system known as social hybridogenesis (Volny and Gordon 2002; Julian et al. 2002; Helms Cahan et al. 2002). This system shows two genetically distinct lineages existing within colonies. Sterile workers are the production of inter-lineage matings whereas new queens are the result of pure lineage matings (Helms Cahan and Keller 2003). Such a system has since been found in *Solenopsis xyloni* (Helms Cahan and Vinson 2003) where colonies are polygynous but each queen is mated with a single male. Queens mated to same-species male produce new queens whereas those mated to

a *S. geminata* male will produce sterile workers. A dependent lineage system has also been found within *Vollenhovia*, *Paratechina* and invasive *Wasmannia* ants where queens are clones of their mother and males clones of their fathers (Fournier et al. 2005; Ohkawara et al. 2006; Pearcy et al. 2011). Finally social hybridogenesis has been observed in populations of *Cataglyphis* desert ants (Leniaud et al. 2012; Eyer et al. 2013; Darras et al. 2014a). Again, workers are inter-lineage hybrids whereas both male and female reproductive are produced by the queen through parthenogenesis such that the male genome is never transmitted to reproductive offspring (Darras et al. 2014b).

Table 1.2 Examples of ants with genotypic variation to caste determination.

Species of ant	Role of genetics in caste determination	Reference
<i>Pogonomyrmex rugosus</i> (variant <i>fuscatus</i>) \times <i>barbatus</i>	Hard-wired genetic control: Queens mate with males of the same lineage become queens; males of a separate lineage are workers (interspecific hybridisation). Two other <i>Pogomyrmex</i> variants have subsequently been shown to exhibit the same phenomenon.	(Helms Cahan et al. 2002; Helms Cahan and Keller 2003) (Volny and Gordon 2002; Julian et al. 2002)
<i>Solenopsis geminata</i> \times <i>Solenopsis xyloni</i>	Interspecific hybridisation as above.	(Helms Cahan and Vinson 2003)
<i>Pogonomyrmex rugosus</i>	Genetic compatibility between maternal and paternal genomes affects queen/worker caste ratio.	(Schwander and Keller 2008)
<i>Linepithema humile</i>	Genetic compatibility between maternal and paternal genomes affects queen/worker caste ratio.	(Libbrecht et al. 2011)
<i>Cataglyphis</i> spp.	Queens produced by asexual reproduction, workers by sexual reproduction.	(Pearcy et al. 2004; Darras et al. 2014a)
<i>Camponotus consobrinus</i>	Genetic component to worker size	(Fraser et al. 2000)
<i>Acromyrmex echinator</i>	Patriline differ in their propensity to develop into specific castes.	(Hughes et al. 2003; Hughes and Boomsma 2007)
<i>Formica selysi</i>	Workers showed heritable components between patrilines in colonies headed by a polyandrous queen and between matrilines in multiple-queen colonies.	(Schwander et al. 2005)
<i>Pogonomyrmex badius</i>	Patriline represented unevenly among worker castes; effects on gyne and major worker size.	(Rheindt et al. 2005; Smith et al. 2008a)
<i>Eciton burchellii</i>	Patrilines represented unevenly among worker castes.	(Jaffé et al. 2007)
<i>Acromyrmex echinator</i>	Some patrilines bias towards queen caste ('royal cheats').	(Hughes and Boomsma 2008)
<i>Atta colombica</i>	Patrilines represented unevenly among castes.	(Evison and Hughes 2011; Holman et al. 2011)
<i>Formica sanguinea</i>	Patriline and matriline effects between worker and gyne/male offspring.	(Haapaniemi and Pamilo 2012)

1.7 Aims of this thesis

This thesis aims to help discover both the ultimate, and proximate, physiological underpinnings of caste and task allocation in both reproductives and workers in ant societies that span a range of social complexities. The predominant focus of the work is carried out in the ‘higher’ leaf-cutting ants of *Acromyrmex* and *Atta*, which show a complex division of labour and a strong worker caste system, where physical size, as well as other environmental and physiological inputs will determine worker task allocation. Comparatively, the more basal, primitive societies of the queen-less, ponerine dinosaur ants offer an insight into the evolution of division of labour at the earliest stages of social lifestyles. Finally this thesis has also utilised alternative ant systems, such as *Messor* harvester ants and other attine species to investigate the role of hard-wired genetic caste determination and the evolution of alarm communication and alarm task allocation within the attine clade respectively. Work such as this, outside of the model system of honey bees is important to help us understand the broader processes and patterns shaping the division of labour, behaviour, and phenotype within these fascinating insects.

2 Behavioural effects of juvenile hormone and their influence on division of labour in leaf-cutting ant societies



Figure 2.1 Accelerating behavioural development. A young (light coloured) brood tender will become a precocious forager following topical application of the juvenile hormone III mimic, methoprene.

2.1 Abstract

Division of labour in social insects represents a major evolutionary transition, but the physiological mechanisms that regulate this are still little understood. Experimental work with honey bees, and correlational analyses in other social insects, have implicated juvenile hormone (JH) as a regulatory factor, but direct experimental evidence of behavioural effects of JH in social insects is generally lacking. Here, we used experimental manipulation of JH, to show that raised JH levels in leaf-cutting ants result in workers becoming more active, phototactic and threat responsive, and engaging in more extranidal activity - behavioural changes that we show are all characteristic of the transition from intranidal work to foraging. These behavioural effects on division of labour suggest that the JH mediation of behaviour occurs across multiple independent evolutions of eusociality, and may be a key endocrine regulator of the division of labour which has produced the remarkable ecological and evolutionary success of social insects.

2.2 Introduction

Division of labour is characteristic of all social groups and is key to the advantages of sociality (Krause and Ruxton 2002). Division of labour can be transient in some species with simple sociality, or can involve behavioural or morphological specialisation for tasks in other species with more complex sociality. The social insects provide classic examples of such division of labour, with division of labour between reproductive individuals and workers being one of their defining characteristics, and with workers also showing division of labour amongst themselves (Oster et al. 1978). The division of labour within social insect colonies is key to their ecological success and results in them

being considered a major transition in evolution (Smith and Szathmary 1997). In some taxa, primarily some ants and termites, division of labour amongst workers is based on morphological castes, while in all social insects it involves behavioural specialisation, often based on age, but often also including division of labour within age classes as well (Hölldobler and Wilson 1990). Understanding the proximate mechanisms which produce the behavioural and morphological division of labour seen in insect societies is therefore one of the key questions in social insect biology, and evolutionary biology more generally.

The environmental and physiological mechanisms involved in division of labour have been extensively studied in the honey bee model organism, in which larvae fed an amino acid-rich diet of royal jelly are stimulated to continue on a queen developmental pathway while larvae receiving a diet lacking in royal jelly instead switch to a worker developmental pathway (Corona et al. 2007; Kamakura 2011). The physiological effect of diet on morphological caste determination in honey bees is mediated by juvenile hormone (JH) levels, with JH also mediating the age polyethism and behavioural task propensity of adult workers (Sullivan et al. 2000; Schulz et al. 2002; Amsalem and Malka 2014). JH has wide ranging effects in insects (Nijhout and Wheeler 1982; Hartfelder 2000; Sullivan et al. 2000; Flatt et al. 2005), and a number of correlational studies have found JH levels to be higher in foragers compared to in-nest workers for several ant, bee and wasp species (Giray et al. 2005; Lengyel et al. 2007; Shorter and Tibbetts 2008; Penick et al. 2011; Dolezal et al. 2012), suggesting that JH may have a general regulatory function in the division of labour in insect societies. However, it is unclear what behavioural changes produce this age-related switch, and direct experimental evidence for the influence of JH outside of the honey bee model system is lacking. Here, we investigated experimentally the behavioural effects of JH in leaf-

cutting ants (which have evolved eusociality independently of bees) by manipulating JH levels. We examined how an increase in JH affects the activity, phototaxis and threat responsiveness of workers, and if it induces workers to engage in precocious extranidal activity, using similar assays to those which have been used in honey bee studies (*e.g.* Erber et al., 2006). We then examine whether the behavioural effects of raised JH levels match those for the key switch in division of labour from intranidal to extranidal work.

2.3 Methods

The experiment used five colonies of *Acromyrmex octospinosus* leaf-cutting ants, a species with large, complex societies of polymorphic workers, in which medium-sized workers care for the brood and mutualistic fungal crop when young, and switch to extranidal foraging and waste management when old (Camargo et al. 2007; Waddington and Hughes 2010). Colonies were collected in Gamboa, Panama, and kept at 80 ± 5 % relative humidity, 26 ± 2 °C, on a 12:12 h light/dark cycle on a diet of privet leaves (*Ligustrum* spp.), with water provided *ad libitum*. Brood-tending workers of medium size (1.71 ± 0.019 mm head width) and young age from within the fungus gardens were collected and treated topically on the pronotum with either 1 μ l of the JH III analogue methoprene (3.3 μ g methoprene in 1 μ l acetone - ca. 0.223 μ g/mg insect mass), or 1 μ l acetone control, three times a week for two weeks. Methoprene is a structural and functional mimic of JH III, binding to the same receptor, although is degraded at a much slower rate, making it an ideal analogue for studying the behavioural or developmental effects of JH III in vivo (Shemshedini and Wilson 1990; Dhadialla et al. 1998; Marrs and Ballantyne 2004). During the application period, ants were housed in individual pots. This methoprene dose was similar to, or lower than, that used previously to produce naturally realistic changes in JH for other social insects (ca. 0.06-0.625 μ g/mg

insect mass per application; Table S3.1) (Shorter and Tibbetts 2008; Helms-Cahan et al. 2011; Tibbetts et al. 2013). Two hours after the last application, ants were tested using the behavioural assays detailed below, with different ants being used for each assay. In order to confirm that ants from the two treatment groups were of similar age, a subset of workers (N= 41 for control, N = 37 for JH) were photographed dorsally and the cuticular darkness (which correlates with age, reliably distinguishing young workers from old; (Armitage and Boomsma 2010)) of the middle third of the rear femur quantified using ImageJ software (Figure S2.1).

2.3.1 Behavioural assays

To test whether methoprene made ants more active, ants were placed individually in a 90 mm Petri dish lined with filter paper, allowed to acclimatise for 5 min, and then filmed for 10 min using a Logitech c920 webcam (methoprene treatment N = 37, control N = 35; 5-6 ants per colony per treatment). Speed of movement was quantified using AntTrak path analysis software (Tranter et al. 2014).

To test whether methoprene affected phototaxis individual ants were placed in a 90 mm Petri dish, with one half blackened out with tape. After 5 min acclimatisation, ants were filmed for 10 min and the proportion of time spent in the light half of the Petri dish recorded (N = 40 for both methoprene and control treatments; 8 ants from each colony per treatment).

To test whether methoprene increased the threat responsiveness of ants, we used a mandible opening response (MOR) assay (Norman et al. 2014). Ants were harnessed and their response to a freshly freeze-killed nestmate or non-nestmate (*Acromyrmex echinator*) worker was tested in a random order. The stimulus was placed in contact

with the focal ant's antennae for 10 s, and its responses were recorded as either mandible gaping for a period of > 1 s (thereby displaying a threat response), or showing no such response during the 10 s (methoprene $N = 37$, control $N = 35$; 7-8 ants per colony per treatment).

To test whether methoprene increased extranidal behaviour in a more natural environment, individuals were observed in a mini-nest set-up, consisting of a small piece of fungus in a 60 mm petri dish, 15 attendant small, young workers to aid fungus care and one larva from the natal colony. The petri dish was covered to keep the nest environment humid and dark, and placed in a larger box (240 mm x 190mm x 75mm). One focal ant from each treatment was placed in each mini-nest and left overnight to acclimatise. The locations of each focal ant were then recorded at 30 min intervals for 8 h (methoprene $N = 38$, control $N = 34$; 6-8 ants per colony per treatment). All assays were then subsequently repeated on 'natural' foragers and nest workers that were observed either within the nest tending to the fungus or outside of the nest collecting leaves, respectively ($n = 30$ for both castes, with 6 ants taken from each of the same five colonies of *A. octospinosus* leaf-cutting ants).

2.3.2 Statistical analyses

All data were analysed in IBM SPSS 20.0 (Chicago, IL, USA) using generalized linear mixed models (GLMM) with treatment (methoprene or control) as a fixed factor and colony-of-origin as a random factor. Model fit was determined using AIC values, with non-significant interaction terms removed to obtain minimum adequate models. The activity (speed of movement), phototaxis (proportion of time spent in the light half of the Petri dish), and behaviour in mini-nests (proportion of time spent engaged in extranidal activity) were analysed with gamma distributions and log link functions, and

threat responsiveness of ants (positive MOR or no response) with a binomial distribution and log link function. Post-hoc pairwise comparisons of treatments were made using the sequential Bonferroni method to control for multiple comparisons.

2.4 Results

Ants treated with methoprene were significantly more active than control ants ($F_{1,70} = 8.77$; $P = 0.004$; Figure 2.2a), and spent significantly more time in the lighter half of the Petri dish instead of the darkened half ($F_{1,78} = 5.87$; $P = 0.018$; Figure 2.2b).

Methoprene-treated ants were also significantly more threat responsive than control ants, exhibiting almost twice as many positive threat responses to both nestmate and non-nestmate stimuli as the control ants ($F_{1,249} = 12.7$; $P < 0.001$; Figure 2.2c). Ants from both treatment groups were also more responsive to a stimulus of a non-nestmate compared to a nestmate ($F_{1,249} = 26.3$; $P < 0.001$). In the mini-nest experiment, young workers treated with methoprene spent on average twice as much time outside the nest environment as control ants ($F_{1,70} = 5.22$; $P = 0.025$; Figure 2.2d). No abnormal behaviours were observed from either control or methoprene-treated individuals.

Treatment and control ants did not differ in their cuticular colouration and therefore age ($F_{1,76} = 0.275$; $P = 0.601$). The effects of methoprene mirror the change seen in leaf-cutting ants when they transition from brood-tending workers to foragers (Figure 2.3).

Foraging workers were significantly more active ($F_{1,58} = 21.2$; $P < 0.001$), more phototactic ($F_{1,58} = 48.9$; $P < 0.001$) and more threat responsive ($F_{1,117} = 9.94$, $P = 0.002$), and spent significantly more time outside of the nest in a mini-nest set-up ($F_{1,1018} = 100.8$; $P < 0.001$) compared to within-nest nurse workers.

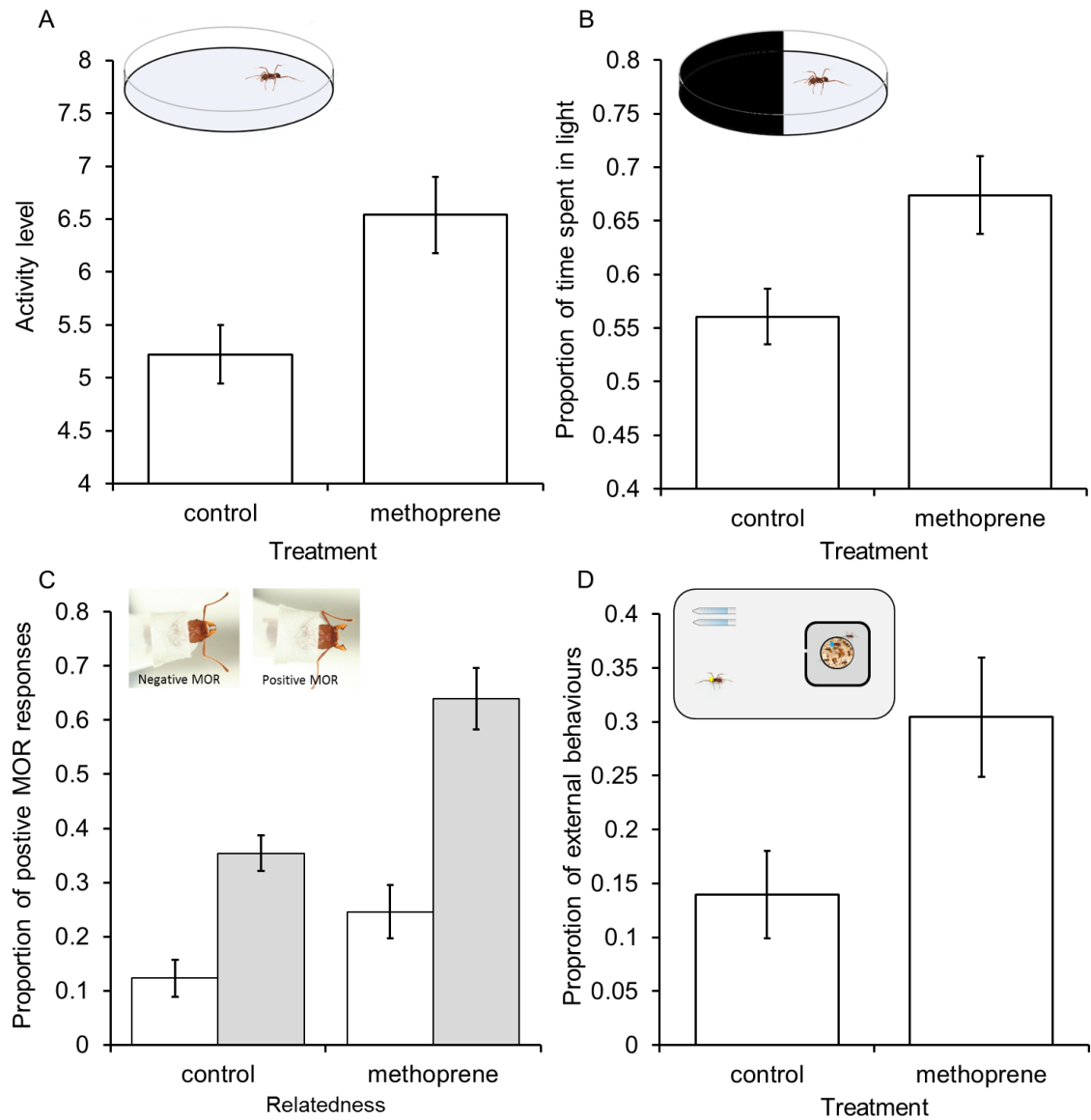


Figure 2.2 Behavioural profile of hormone treated in-nest workers. The mean \pm s.e.m. of behavioural variables measured for young leaf-cutting ant workers from five *Acromyrmex octospinosus* colonies that had been treated for 2 weeks with either the JH analogue methoprene or solvent control. Ants treated with methoprene were (A) were significantly more active than solvent only, control ants (N = 37 for methoprene, N = 35 for control), (B) spent significantly more time in the lighter half of the Petri dish instead of the darkened half (N = 40 for both methoprene and control), (C) were significantly more threat responsive to both a nestmate (white bars) and a non-nestmate (grey bars) than control ants (N = 37 for methoprene treatment, N = 35 for control) and (D) spent significantly more time exhibiting extranidal activity than control ants (N = 38 for methoprene, N = 34 for control).

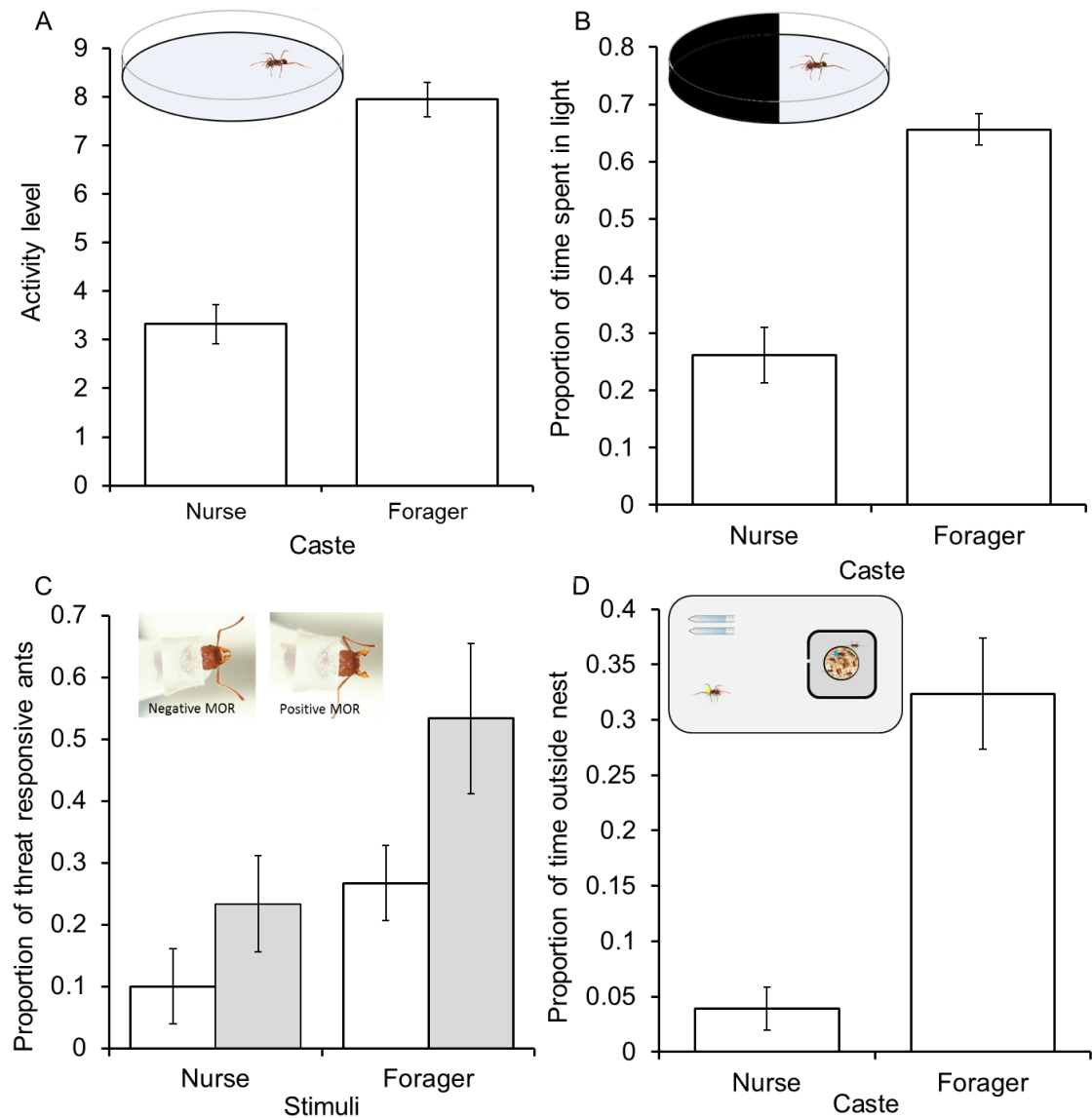


Figure 2.3 Behavioural profile of untreated foragers and in-nest workers. The mean \pm s.e.m. activity for leaf-cutting ant workers that were either foragers (N= 30) or in-nest workers (N = 30) from five *Acromyrmex octospinosus* colonies. Foragers were (A) significantly more active (B) phototactic and (C) threat-responsive to both a nestmate (white bars) and a non-nestmate (grey bars) and (D) showed significantly more extranidal activity compared to in-nest workers.

2.5 Discussion

The results show that JH level can strongly affect the behavioural profiles of ant workers, and induce them to engage in precocious extranidal activity. Treatment with the JH analogue methoprene caused a significant increase in activity, phototaxis, and threat responsiveness of leaf-cutting ant workers, all of which are behavioural

characteristics of foraging in the study species, as well as social insects in general (Chapman et al. 2011; Pamminer et al. 2014). Furthermore, there was a doubling in the amount of time that methoprene-treated workers spent engaging in extranidal activities in a mini-nest setting compared to control ants. JH therefore affects the switch between intranidal and extranidal work that is a key part of the division of labour in ant societies, and it does this by making ants more active, phototactic and threat responsive. This therefore provides evidence for a ‘toolkit’ situation involving JH. This is indicated by the recruitment of JH multiple times independently as a hormonal ‘tool’ to regulate the division of labour which we observe both in bees and now in ants which have evolved eusociality separately to bees. Such findings indicate that JH regulation of behaviour is either ancestral in eusocial Hymenoptera or has evolved multiple times.

The results are, to our knowledge, the first direct experimental evidence of the effect of JH on division of labour in ant societies, and of the behavioural mechanisms by which this effect is produced. The effects of JH observed on leaf-cutting ants have remarkable similarities with those seen in the best-studied social insect, the honey bee *Apis mellifera*, in which experimentally elevated JH levels lead to precocious foraging (Sullivan et al. 2000), possibly due to the same behavioural mechanisms as we show here in leaf-cutting ants. The results also fit with a number of correlational studies in which JH levels have been found to be higher in foragers of several different ant species, as well as wasps, bees and termites (Giray et al. 2005; Lengyel et al. 2007; Dolezal et al. 2009; Penick et al. 2011). This suggests that JH may have a highly conserved role as a key endocrine mediator of division of labour within eusocial insect societies that has been key to their ecological and evolutionary success.

3 The ambiguous role of juvenile hormone in regulating reproductive physiology but not dominance hierarchy in *Dinoponera quadricaps*

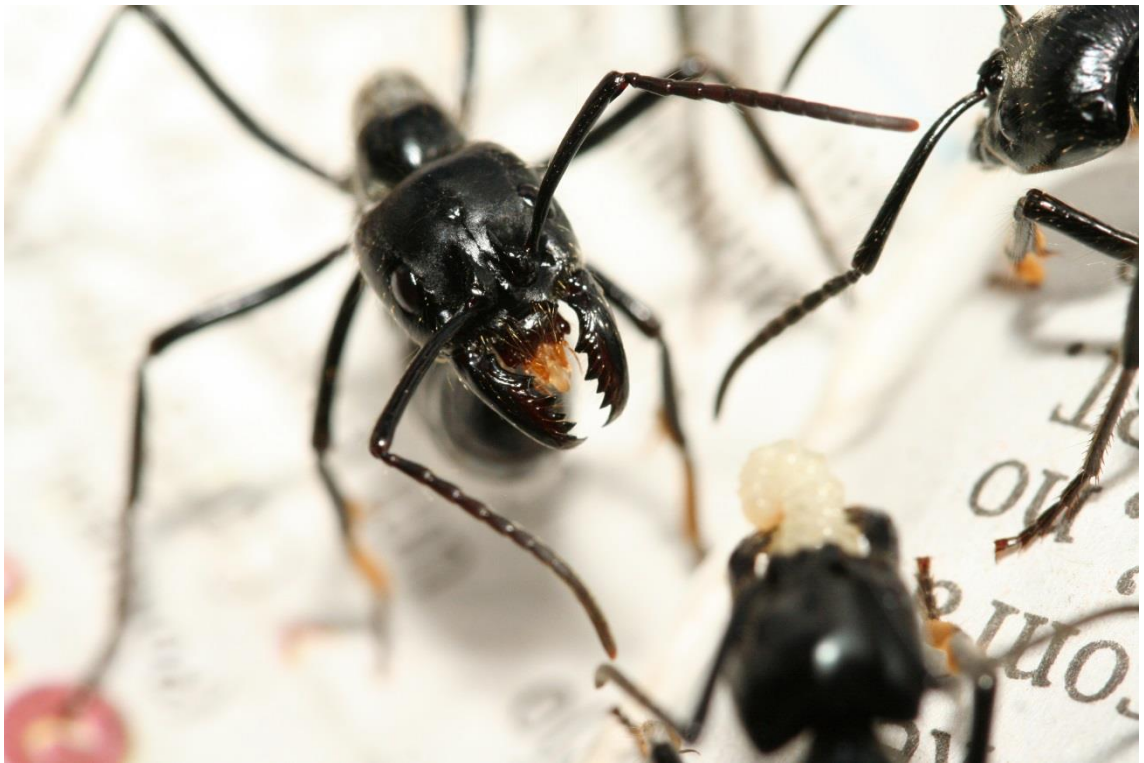


Figure 3.1 Dominance hierarchies in primitively social dinosaur ants *A Dinoponera quadricaps* individual shows ritualised, aggressive behaviours towards a subordinate nestmate.

3.1 Abstract

Unequal reproductive output among members of the same sex (reproductive skew) is a common phenomenon in a wide range of communally breeding animals. In such species, reproductive dominance is often acquired during antagonistic interactions between group members that establish a reproductive hierarchy in which only a few individuals reproduce. Rank-specific syndromes of behavioural and physiological traits characterize such hierarchies, but how antagonistic behavioural interactions translate into stable rank-specific syndromes remains poorly understood. The pleiotropic nature of hormones makes them prime candidates for generating such syndromes as they physiologically integrate environmental (social) information, and often affect reproduction and behaviour simultaneously. Juvenile hormone (JH) occupies such a central regulatory role in insects and has been suggested to regulate reproductive hierarchies in a wide range of social insects including ants. Using JH manipulation of high ranked workers in the ant *Dinoponera quadriceps*, a queenless species with simple societies, we show that in contrast to our expectations, JH regulated reproductive physiology but lacked any regulatory function for dominance related behaviour. There was therefore no evidence that JH is involved in generating rank associated trait syndromes or is causally linked to hierarchy establishment and maintenance in this species. This surprising result stands in clear contrast to the suggested function of JH as a key regulator of reproductive hierarchies and highlights the regulatory flexibility of JH in adult insects. We discuss the evolutionary consequences of the decoupling of reproductive and behavioural control in *D. quadriceps* and the surprising flexibility of regulatory functions of JH.

3.2 Introduction

In many group-living animals, reproduction is not equally distributed among its breeding members. This phenomenon, known as reproductive skew, occurs in a wide range of communally-breeding species including birds, fish and insects (Jamieson 1997; Cuvillier-Hot et al. 2004a; Neff et al. 2008). Due to its fundamental implications for both ecological and evolutionary processes, this topic has attracted attention over the past decades from both a theoretical (see Kokko and Johnstone 1999; Johnstone 2000; Kokko 2003) and an empirical perspective in a variety of study systems (Bourke et al. 1997; Field et al. 1998; Reeve and Keller 2001; Widdig et al. 2004).

Social insects, in particular ants, have emerged as important model systems to test some of the main predictions of reproductive skew theory due to their wide range of social complexity and life history strategies (Reeve and Keller 2001). In the majority of ant species, a single queen produces all female offspring and her unmated daughter workers perform all other tasks such as brood care, foraging and nest defence (Hölldobler and Wilson 1990). In such systems, as a result of the haplodiploid sex determination in Hymenoptera, workers are only able to produce male offspring and conflicts between queens and workers arise over male parentage (Ratnieks and Reeve 1992; Ratnieks et al. 2006). This group conflict and how it is resolved has generated a plethora of ground-breaking work, revolutionizing our understanding of group formation, conflict and maintenance (for a review see Ratnieks et al., 2006). While most modern ants have a specialized queen caste, some genera have secondarily lost queens, with reproduction being monopolized instead by mated, reproductively active workers called gamergates (Peeters 1997). In most gamergate systems, all workers have the potential to become the dominant reproductive, resulting in strong within-group conflict

over reproduction (Peeters, 1997). Such conflicts are often resolved via aggressive behavioural interactions that establish a dominance hierarchy in which only a single, or a small group of workers go on to reproduce.

In *Dinoponera quadriceps*, a ponerine ant species from Brazil, one dominant gamergate, the alpha, actively suppresses a group of the higher ranked workers from becoming reproductively active with ritualized physical aggression including antennal blocking and boxing (Monnin and Peeters 1998; Grainger et al. 2014). The presence of an alpha within the colony not only inhibits ovary activation in workers, the first step towards becoming reproductively active, but also results in submissive behaviour by subordinates (Smith et al. 2011; Asher et al. 2013). The phenotypic differences between alphas and subordinates result from subtle differences in transcriptional network organisation, involving both conserved and novel genes (Patalano et al. 2015). The question of how such ritualized physical aggression is physiologically translated into stable reproductive hierarchies with lower ranked workers not only remaining reproductively inactive but also assuming helper roles, remains poorly understood.

Hormones are prime candidates for the proximate mechanisms underlying this process, because they not only physiologically integrate social stimuli including stress, but also regulate numerous other essential processes in adult insects such as reproduction, maternal behaviour and aggression (Nijhout 1998). It has been shown in gamergate ants of the genus *Streblognathus* and *Diacamma* that low JH titres correlate with high individual ranks within the hierarchy, and that JH application will result in a loss of the reproductive status of the alpha (Brent et al., 2006; Cuvillier-Hot et al., 2004). However this seems to not generally be the case as in some higher (non-gamergate) ant genera, namely *Solenopsis* and *Pogonomyrmex*, JH exhibits stimulatory functions during reproduction (Brent and Vargo 2003; Libbrecht et al. 2013). In

addition JH is known to trigger foraging behaviour in some social insects, making it a possible candidate to coordinate not only reproductive division of labour, but also division of labour between workers (Robinson and Vargo 1997; Norman and Hughes 2016).

Here we use topical application of the JH analogue (JHa) methoprene to high-ranked workers, with the potential to lose their rank and potential future reproductive status, to investigate if JH affects reproductive physiology, individual behaviour and consequently position in the hierarchy. If JH links reproduction and hierarchy-related behaviours it would then provide a proximate physiological explanation for rank-associated trait syndromes.

3.3 Methods

3.3.1 Study organisms

We investigate the role of JH in maintenance of the dominance hierarchy in the monomorphic, queenless, ponerine ant species *Dinoponera quadriceps*. This species is of particular interest as it is one of only a few species to have undergone an evolutionary reversion from a highly eusocial ancestor with a queen caste back to its basal, primitively social, queenless state (Monnin and Peeters, 1998; Peeters, 1997). We used 11 colonies of *D. quadriceps*, which were collected from Bahia state, Brazil in November 2014. All colonies were maintained in the lab at 27°C and 80% relative humidity for at least six months before the experiment. Colonies were fed with *Tenebrio molitor* larvae and apple, and had *ad libitum* access to water. Each individual was uniquely marked on the pronotum with numbered tags.

3.3.1.1 *Establishing the dominance hierarchy*

Firstly, to establish the dominance hierarchy, colonies were monitored daily for two weeks, with the behaviours and locations of each individual being recorded. Individuals showed high levels of consistency in behaviour and location during this period. Given the positive association in this species between an individual being of high rank and it interacting with brood (Monnin and Peeters 1999; Asher et al. 2013) any individual that was observed at least once interacting with brood was selected to undergo pairwise isolated dyadic interactions. This method pairs every combination of ants sampled to observe which individual in each dyad is the dominant and which the subordinate, based on a characteristic dominance behaviour; this has previously been shown to be a reliable and robust way to establish dominance hierarchies in this species (Grainger et al. 2014). For this, individuals were taken from their colonies and placed individually in pots (85 mm x 75 mm x 55 mm) and allowed to acclimatise for 15 min. Pairs of ants were then placed in a new pot, their dominance interaction observed and the dominant ant recorded. This is indicated by only one behaviour in this context: dominant ants stand tall with their antennae either side of the subordinate individual which has antennae laid flat back behind their head (Grainger et al., 2014). This reaction normally occurs within the first 60 s of contact between pairs when it is expressed. We then ranked individuals based on the number of times they expressed dominance, and assigned ranks to each individual. The three individuals that ranked directly below the alpha (the highest ranker) were then selected for the study.

3.3.1.2 *Worker size and weight*

Before the start of the experiment all selected workers were immobilized on ice for 1 min and their head width (maximal interorbital distance) measured as proxy for body size, as well as their fresh weight using a Precisa 125A balance.

3.3.2 Behavioural measures and experimental procedure

Behavioural observations were made for five days before the first application of treatments to determine how consistent ants were for a number of behavioural variables. We carried out daily scans for five days prior to treatment in which we recorded for each focal ant whether or not it was showing any aggression (either within the nest to conspecifics or gaping its mandibles in defence outside of the nest), whether or not it was showing any brood care behaviours, and two measures of ‘sociability’: the distance to the nearest ant and the number of ants within 5 cm of the focal ant. During the same time interval we carried out individual-level assays for activity level, phototaxis and aggression, with the expectation that high rankers would show low activity level (as higher rankers perform fewer tasks than lower rankers (Monnin et al. 2003)) low phototaxis (as they are based inside and away from any ‘risky’ tasks such as nest defence or foraging (Nascimento et al. 2012; Asher et al. 2013)) and high levels of aggression (known to be associated with higher ranks (Monnin and Peeters 1999; Cant et al. 2006)). Activity level was determined by placing the focal ant in a 90 mm Petri dish lined with filter paper, leaving it to acclimatise for 2 min, and then videoing the ant for 5 min using a Logitech c920 webcam. Speed of movement was quantified from videos using Antrak path analysis software (Tranter et al. 2014). Phototaxis was determined by placing the focal ant in a 90 mm Petri dish lined with filter paper and half blackened out with tape, leaving it to acclimatise for 2 min, and then videoing the ant for 5 min to allow the proportion of time spent in the light half of the Petri dish to be calculated. Aggression was determined by placing the focal ant in a pot (85 mm x 75 mm x 55 mm), leaving it to acclimatise for 5 min, and then tapping it gently on the head with the tip of a toothpick, as in Pamminer et al. (2014). The reaction of the ant was ranked (0 = ignore, 1 = antennate, 2 = gape mandibles in a threat response, 3 = bite).

Following the initial assessment of individual behaviour, ants were assigned randomly to either the methoprene treatment or solvent control, and all subsequent behavioural observations were conducted with the observer blind to the treatment. For the methoprene treatment, ants a dose of 16.5 µg of methoprene (PESTANAL[®] Sigma Adrich[®]) in 5 µl acetone was applied to the pronotum three times over a period of 1 week; control ants received 5 µl acetone on the same occasions. This dose was determined during a preliminary experiment and is low compared to the amounts used in other social insect studies (Table S3.1), indicating that the observed effects are not caused by potential toxic effects of JH at high doses. After two days of acclimatisation post-treatment, we repeated the behavioural observations. We carried out the assays daily for 4 days and on the 5th day carried out dyadic interaction assays between the focal ants and all other workers that had been observed performing brood care behaviour over the past three weeks. This enabled us to determine if the methoprene treatment had not only affected behaviour but also the position of the focal high rank ants in the hierarchy. Following the dyadic interactions, ants were freeze-killed in liquid nitrogen and stored at -80 °C until ovary dissection.

3.3.3 Ovary dissection and fertility estimates

Ant ovaries were dissected under a Leica S8AP0 stereo microscope and the ovaries were transferred into Ringers solution. The ovaries were photographed using a Leica DFC 295 Camera and the Leica application suite software v. 4.1.0. Three ovarioles were randomly selected for further analysis. Using a Pyser-SGI[®] S78 stage micrometer 1.0/0.01 mm and the software ImageJ 1.47v, we measured the minimum, maximum and average width of the most distal third of the ovarioles (containing the furthest developed

eggs if present) and the number of vitellogenic eggs, which are the white (yolk), non-transparent and non-deformed portion of the eggs found in the ovarioles.

3.3.4 Statistical analysis

For the statistical analysis, we used the programme PRIMER 6, version 6.1.13, + add-in, version 1.0.3 (PRIMER-E Ltd) to perform permutational multivariate analysis of variance (PERMANOVA). PERMANOVA is a non-parametric MANOVA, which has the advantage that it is free from assumptions on data distributions (Anderson et al. 2008). All tests were carried out using 9,999 permutations on a resemblance matrix using Euclidian distance as a distance estimates. In all cases we used treatment as a fixed factor and colony as a random predictor variable to account for the structured nature of the data. Interaction between the factors was included, but removed from the final minimum adequate model when nonsignificant. All response variables were z-transformed prior to analysis in order to account for difference in units and variation between variables, which facilitates the interpretation of results in particular interactions between variables (Gotelli and Ellison 2004).

To test for potential differences in weight and size between workers belonging to different colonies and treatments, both were used as response variables in a PERMANOVA. To investigate behavioural response to treatment we used the change in behaviour following JH treatment as response variables for analysis. We calculated the mean behaviour and hierarchy position (number of winning, dominant encounters) before and after treatment to obtain a robust estimate for brood care, aggression, phototaxis, activity and sociability (ants in close proximity and distance to the nearest ant) and position in the hierarchy before and after treatment. We then calculated the change in behaviour in response to treatment by subtracting the averaged behaviour

value before treatment from the average value after treatment; positive values therefore indicate an increase, and negative values a decrease, in response to treatment. The same calculation was performed for the change in rank (number of encounters won in dyadic interactions). We used minimum, maximum and average ovary width and the number of vitellogenic eggs as response variable in the fertility analysis. To further analyse the qualitative differences between the treatments, we performed a one-way similarity of percentage (SIMPER) analysis, a data exploration technique that calculates the contributions individual factors make to both group (treatment) coherence and separation in a non-metric multidimensional scaling (MDS) analysis. We also carried out a further analysis just comparing rank changes between treatment group over the course of the experiment. Here, change in rank was used as a response variable in a Generalized Linear Mixed Effects Model (GLMM), with colony of origin as a random factor, treatment as a fixed factor specifying a multinomial distribution and a cumulative logit link function.

3.4 Results

Ants did not differ in terms of size or weight between colonies (Pseudo $F_{11,31} = 1.04$, $P = 0.4$) or treatments (Pseudo $F_{1,31} = 0.35$, $P = 0.72$). We also found no significant differences between colonies (Pseudo $F_{11,31} = 1.37$, $P = 0.06$) or treatment (Pseudo $F_{1,31} = 0.74$, $P = 0.6$) in behaviour or hierarchy position (Figure 3.2). However, while worker fertility did not differ between colonies (Pseudo $F_{11,31} = 1.82$, $P = 0.11$), it was strongly affected by treatment, with JHa-treated individuals being less fertile compared to the control acetone (CoA) group (Pseudo $F_{1,31} = 6.87$, $P = 0.015$; Figure 3.3). The CoA group was more coherent when compared to the JHa group (Table 3.1; Figure 3.3), as indicated by the low within-CoA average squared distance of 0.92 compared to the 6.39

in the JHa group. Furthermore, there were large differences in fertility within the JHa group (Figure 3.3). When looking at the factors contributing to group separation, we find that all markers of fertility (number of vitellogenic oocytes and size of ovarioles) contribute equally to group separation (Table 3.1), with CoA exhibiting subaverage values in all categories. In a separate analysis just focussing on rank, although on average both treatments lost rank over the course of the experiment there was no significant difference in rank lost between JHa and CoA treatment groups ($F_{2, 24} = 0.98$, $P = 0.39$; Figure 3.4).

Table 3.1 Results of the fertility SIMPER analysis. Presenting the average distance between samples both within and between treatments and the contribution of the individual factors in per cent

	JHa	CoA	Between CoA and JHa
Average squared distance	0.92	6.39	9.32
Trait contribution			
Separation %			
Oocyte number	12.95	21.06	25.73
Ovariole minimum width	13.86	25.79	25.59
Ovariole maximum width	14.45	25.92	25
Ovariole average width	58.74	27.24	26.68

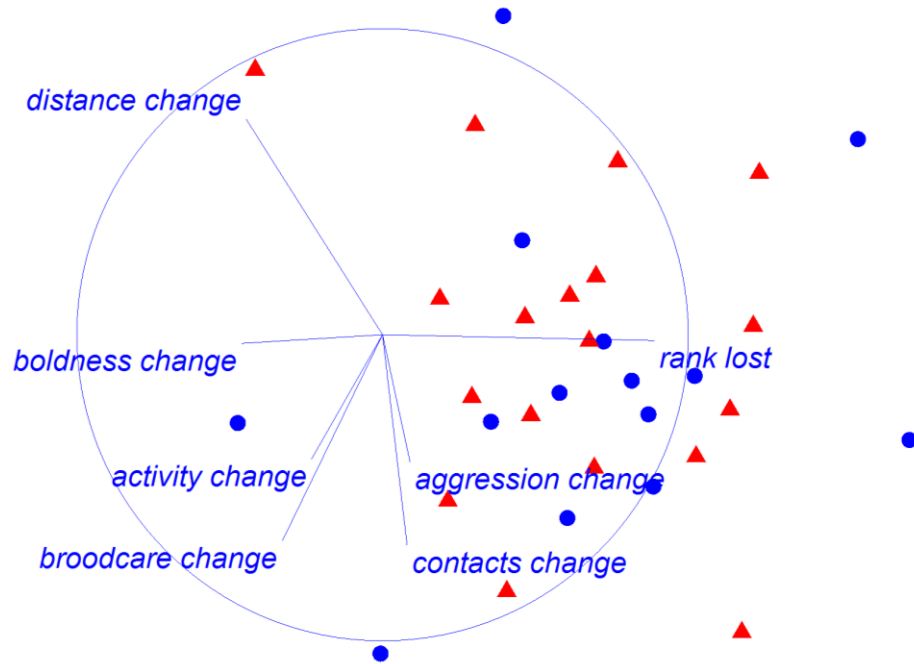


Figure 3.2 Multidimensional scaling (MDS) plot of all behaviours measured. Vectors indicate strength and contribution of the individual traits for group separation between the two treatment groups (CoA = blue circles, JHa = red triangle). There were no significant differences between the treatments.

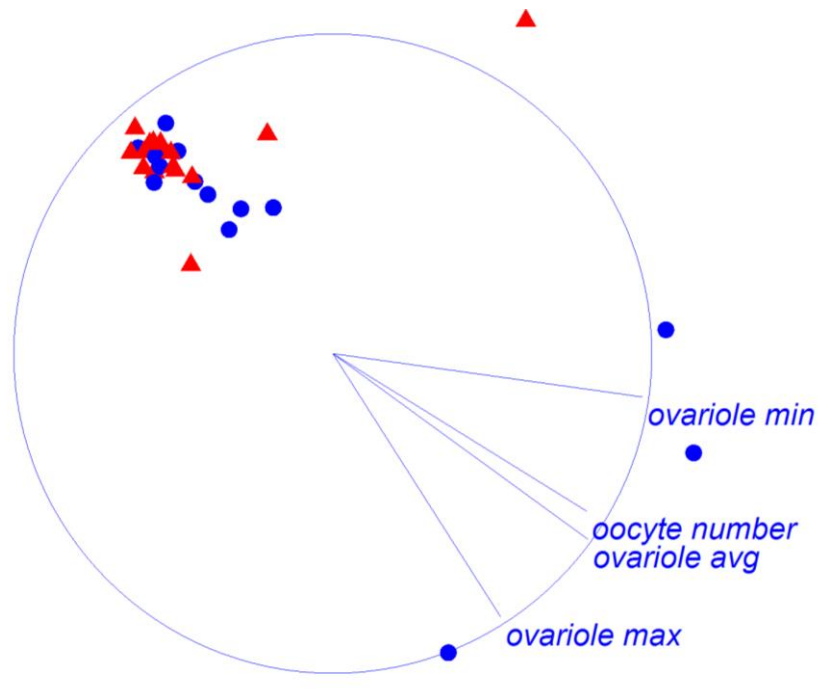


Figure 3.3 Multidimensional scaling (MDS) plot of all fertility estimators measured. Vectors indicate strength and contribution of the individual traits for group separation between the two treatment groups (CoA = blue circles, JHa = red triangle). CoA-treated ants exhibited significantly higher ovary activation compared to JHa-treated ants.

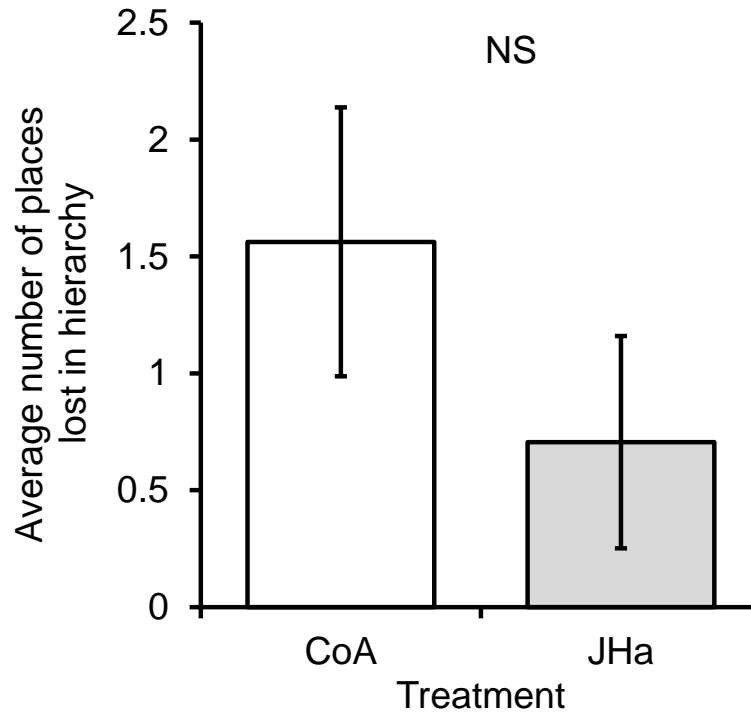


Figure 3.4 Average rank lost between methoprene treated (JHa) and control treated (CoA) dinosaur ants All ants lost rank over the course of the experiment and, although hormone treated ants showed a general trend for a slightly larger loss there was no significant difference between treatment groups in rank change in the hierarchy over the two week application period.

3.5 Discussion

Our results show that JH occupies a central role in regulating reproduction in *D.*

quadricaps. JH not only decreases the number of vitellogenic eggs, but also results in an overall decrease in the size of individual ovarioles indicating a substantial reduction in reproductive potential. In solitary insects JH often has the opposite effect by stimulating the production of vitellogenic oocytes and the same is true for many primitively eusocial insects, such as non-swarm founding wasps and bumblebees (Robinson and Vargo 1997). It has been suggested that the functional reversal of JH in reproduction was important in the transition to eusociality and consequently social complexity, and a large number of studies demonstrate often radical changes in the regulatory architecture of reproduction in eusocial species (Robinson and Vargo 1997; Hartfelder 2000; Bloch

et al. 2009). The classic example for this argument is the function reversal of JH in honeybees and some ants (Robinson and Vargo 1997; Bloch et al. 2009; Azevedo et al. 2016; Pamminer et al. 2016), however a small number of studies clearly indicates that high social organization is possible without it (e.g. Brent and Vargo 2003; Kelstrup et al. 2015). *D. quadricaps* clearly supports these findings by demonstrating that a reversal of JH function in reproduction can be associated with simple social organization. Given *D. quadricaps*' evolutionary position of a reversal to a more simple society from a highly advanced ancestor we observe more similarities in terms of JH's effects on reproduction with such advanced societies rather than compared to a truly primitive society suggesting a different role for juvenile hormone between primitive and advanced social species. This further supports the notion that there is likely no causal link between the remodelling of the JH function in reproduction and the organisational complexity of insect societies.

In contrast to the relatively well-studied effects of JH on reproductive physiology, little is known about the regulatory role of JH in behaviour for most insects. The association between JH and aggression, maternal behaviour and activity has been documented in insects (Nijhout 1998; Pearce et al. 2001; Tibbetts and Izzo 2009; Tibbetts et al. 2013), however these studies are restricted to only a handful of species. In the honey bee *Apis mellifera*, JH, in combination with the yolk pre-cursor vitellogenin, regulates one of the major behavioural transitions in the adult honeybee worker from within-nest behaviour to external foraging (Robinson and Vargo 1997). This transition is associated with a major remodelling of the behaviour repertoire and indicates the far-reaching regulatory potential of JH in behaviour. A similar function of JH has been documented in *Pogonomyrmex californicus* harvester ants and *Acromyrmex echinator* leaf-cutting ants (Dolezal et al. 2009; Norman and Hughes 2016), demonstrating that JH

can generate forager-like behavioural phenotypes. In contrast to our expectations, we did not find here any measurable effects of JH on worker behaviour or position in the hierarchy in *D. quadricaps*. Lower ranked workers preferentially perform the foraging tasks in this species (Monnin and Peeters 1999; Asher et al. 2013), so consequently our study provides no evidence for JH regulating the tasks a worker performs in this species. It is possible that the strict reproductive hierarchy in *D. quadricaps* might inhibit behavioural changes of the individual workers; however our behavioural assays were conducted outside the colony context and are consequently unlikely to be directly influenced by social interactions. It would be an interesting follow up experiment to repeat this experiment after removing the dominant reproductive individual and investigate if JH influences the establishment of a new reproductive hierarchy.

The results suggest that reproductive capability and behavioural phenotype may therefore be decoupled in *D. quadricaps*, and that JH is not directly involved in maintenance of the reproductive hierarchy by ritualized aggressive behaviour. If it is the most behaviourally dominant individual that becomes the gamergate (Monnin and Peeters 1998), rather than the most fertile individual, then this could result in a fitness loss at the colony level if the most aggressive individual is not also the most fertile. Alternatively, it might be possible that JH still has some long-term function in regulating behaviour in *D. quadricaps* or the dose required to observe behavioural alterations might differ from those required for ovary degradation. However, a similar lack of behavioural alteration following JHa application was observed in alphas of *Ectatomma* ponerine ants (Cuvillier-Hot et al. 2004a), making lack of function in regulating behaviour the most likely explanation and suggesting that this might be a more common effect in gamergate systems. This further supports the argument that JH has a different role in truly primitive and advanced societies, and with these ponerine

gamergate systems being a derived state from an advanced eusocial ancestor it is likely that JH has a similar effect in such systems as it does in ‘higher’ systems of ants and bees (Robinson and Vargo 1997; Bloch et al. 2009; Azevedo et al. 2016).

This lack of regulatory function in behaviour suggests that JH is unlikely to be responsible for the generation of rank-associated trait syndromes linking reproductive status and associated behaviour in *D. quadricaps*. Consequently other hormones like ecdysone or vitellogenin might be responsible for generating the observed rank-specific phenotypes and the maintenance of the reproductive hierarchy (Hartfelder 2000). Further work combining behavioural, genetic and physiological work is needed to illuminate the regulatory underpinning of reproductive hierarchies in simple ant societies. When looking at the broader phylogenetic picture there is accumulating evidence that JH occupies a stunning range of different, often opposite, regulatory functions. The question how such incredible regulatory flexibility is possible without compromising fitness relevant functions is intriguing and a promising target for further molecular and comparative investigations.

4 Old and wise but not size: factors affecting threat response behaviour and nestmate recognition in *Acromyrmex echinator* leaf-cutting ants



Figure 4.1 Nestmate recognition in the leaf-cutting ant *Acromyrmex echinator*. A large *A. echinator* individual recognises a small intruder from a different colony.

4.1 Abstract

Detecting and responding to threats is of prime importance for social species which need to be able to distinguish nestmates from intruders to protect the resources of their colony. However individuals may differ in their propensity to recognise threats due to factors, often intercorrelated, such as caste, age and experience and the ability to separate these is important for understanding why behaviours are expressed. Here, we use leaf-cutting ants in a controlled behavioural assay to tease apart the factors which likely affect threat response behaviours in social insect workers. We show that foraging workers respond to threats more readily than do within-nest workers. The response of all workers was greater towards more foreign stimuli – nestmates rarely stimulated a response, whereas ants of a different genus stimulated a response in most cases. We show that age and experience act separately to increase an individual's ability to perceive the threat. This suggests that where multiple, compounding factors affect the expression of certain behaviours it is important to realise that these factors can also have independent effects, particularly those which correlate with age. Separating the influence of correlating factors experimentally, as shown here, is particularly useful for understanding why individuals may differ in their behavioural profile.

4.2 Introduction

In group-living organisms, the defence of shared resources is of vital importance to group security. Such resources may include, shelter, nesting habitat, food or the individuals themselves, particularly those that contribute more towards the reproduction of the group (Hölldobler and Wilson 1990; Seeley 2009; Wenseleers et al. 2013). The first step in any defence is for individuals to be able to recognise a threat and

communicate this to fellow group members. Without this stage, the group as a whole will not be able to mount a suitable response, be it fight or flight (Verheggen et al. 2010). While the ability of organisms to recognise nestmates from non-nestmates has been much addressed (e.g., Sturgis and Gordon, 2012a), few studies have disentangled the various interacting processes influencing response behaviours. Here, we investigate these processes under controlled laboratory settings.

Social insects in particular have evolved efficient recognition systems whereby workers can detect and subsequently defend their colony from external threats such as robbery or parasitism (Gamboa 1978; Walker and Hughes 2009). Division of labour within social insect colonies is well known (Oster et al. 1978; Wilson 1990; Robinson 1992), and this also extends to workers differing in their propensity to perform specific nest defence behaviours. Examples vary from species with a morphologically specialised soldier caste (Grüter et al., 2012), to behaviourally specialised guard workers (Butler 1952; Moore et al. 1987). However, the ability of workers outside these task groups to perceive threats is also crucial to the colony being able to respond to a threat quickly and appropriately. The recognition of nestmates from non-nestmates in most social insects is thought to be based on cuticular hydrocarbons (Bonavita-Cougourdan et al. 1987; Wagner et al. 2000; Liang and Silverman 2000), although colony differences in composition and behavioural bioassays suggest that alarm pheromones may also be informative for nestmate recognition in some taxa such as leaf-cutting ants (Brandstaetter et al., 2008; Francelino et al., 2006, 2008; Hernández et al., 2006; Hughes et al., 2001; Whithouse and Jaffe, 1996).

There are many factors which may affect an individual's propensity to exhibit threat response behaviour, but being able to independently test each factor can be challenging. Important factors in social insects include size (Nowbahari et al. 1999;

Huang 2010; Hölldobler and Wilson 2010) and age (Seeley 1982; Morel et al. 1988; Jaisson 1991; Waddington and Hughes 2010) with, for example, larger ants being expected to act more aggressively or older ants to have a more developed ability to distinguish between nestmate and non-nestmate. Experience has also been shown to be a contributory factor in some aspects of nestmate recognition (Beshers and Fewell 2001; van Wilgenburg et al. 2010). The difficulty, however, is that age and experience are often correlated and few studies have looked at whether these factors act separately. Laboratory colonies enable these factors to be separated. In this setting older ants are no more experienced in interacting with foreign individuals than younger ants, something which is not necessarily the case in field colonies.

Studies regarding nestmate recognition often use aggression to score how successful an individual is at discriminating friend from foe (*e.g.*, D’Ettorre et al., 2006; Downs, 2000; Guerrieri et al., 2009; Kikuchi et al., 2007; Van Wilgenburg et al., 2010). Whilst aggression is indicative of the detection and rejection of a non-nestmate, it is just one part of the nestmate recognition process; individuals may perceive the threat but exhibit a different behaviour, such as an alarm or panic response (Verheggen et al. 2010). However, in all of these responses individuals open their mandibles to release alarm pheromone from the mandibular glands and to prepare to bite (Wilson and Regnier 1971; Stoeffler et al. 2007). This makes the mandible opening response (MOR) an excellent indicator of threat detection and responses in general because it is expressed during aggression, alarm and panic rather than solely aggression (Guerrieri and D’Ettorre 2008). This specific mandible opening behaviour indicates that a threat has been perceived by the focal ant (Hölldobler and Wilson, 1990; Hughes et al., 2001), making it highly biologically relevant for studies concerning how and why individuals differ in their tendency to recognise threats. Such threats to leaf-cutting ant colonies

could include vertebrate predation attempts (such as by armadillos) for the nutritiously dense fungus garden, predominantly responded to by larger ants, or soldiers in *Atta* leaf-cutting ants (Wilson and Hölldobler 1990; Whitehouse and Jaffe 1996). A second type of attack would be from conspecific or intraspecific threats, for example in competition of over food resources, where smaller ants are more heavily recruited to combat the threat (Wilson 1980; Hughes and Goulson 2001; Hölldobler and Wilson 2010). The MOR is, in some ways, similar to the proboscis extension response and sting extension response used in honey bees, in being an assay allowing an objective evaluation using a categorical response (yes/no) (Page et al. 1998; Balderrama et al. 2002). It allows ants to be tested individually, thus avoiding trials involving multiple individuals at once which may confound and complicate the response of individuals (Kikuchi et al., 2007; Pamminer et al., 2011; Sturgis and Gordon, 2012b).

Here, we use the MOR assay to tease apart the importance of task, caste, age and experience in threat response behaviour in a controlled and standardised way. We study the propensity of workers from the leaf-cutting ant *Acromyrmex echinator* to exhibit a threat response behaviour to non-nestmates in a number of contexts (assessing the roles of cuticular hydrocarbons versus alarm pheromone in recognising a non-nestmates from nestmates), and test the importance of task, caste, age and experience on how this behaviour is expressed.

4.3 Methods

Eight *A. echinator* colonies were used for Experiment 1 (Ae1103, Ae1102, Ae088, Ae399, Ae1003, Ae396, Ae603, Ae084) and six for Experiments 2, 3 and 4 (Ae1102, Ae1105, Ae399, Ae088, Ae396, Ae603), all collected from Gamboa, Panama between

2008 and 2011. Colonies had been kept in the laboratory for at least two years before the experiments were conducted. Colonies were kept at 80 ± 5 % relative humidity, 26 ± 2 °C and 12:12 h light/dark cycle. All fungus chambers were housed in sealed plastic nest boxes. They were fed twice weekly on privet leaves (*Ligustrum* spp.) placed in a foraging pot (c. 100 mm x 80 mm x 60 mm) and provided with water *ad libitum*.

4.3.1 Mandible opening response assay

The MOR assay was conducted following Guerrieri and d'Ettorre (2008). Ants were cooled on ice until immobilised and were then harnessed, leaving only the head, antennae, and mouthparts free to move. The harness was made using a 0.2 ml pipette tip (Starlab, Bucks, UK), cut at the apex through which the ant's head was passed and secured with a thin strip of masking tape. Ants were left for 2 h to acclimatise to the harness and recover from the anaesthesia before experiments began. There was no mortality over the 2 h period. All experimental treatments were presented in a random order to each subject with at least a 5 min interval between stimuli. Each stimulus was presented to the ant for a 20 s period. Ants occasionally opened their mandibles very briefly (< 1 s) when any object contacted their antennae, so we only recorded a MOR if the ant opened its mandibles widely for a period of > 1 s.

4.3.2 Experiment 1: does threat response behaviour differ between within-nest workers and foragers?

Foragers were removed from the foraging pot and nest workers were taken from the fungus garden after being observed tending to the fungus. Between four and six ants of each caste were taken from each of the eight colonies, giving a total of 38 foragers and 42 within-nest workers. The cuticle of leaf-cutting ant workers darkens with age

(Armitage and Boomsma 2010), and individuals at the extreme ends of the range of cuticular colouration were avoided to minimise differences in ages between ants. Each ant was presented with the following five stimulus types in a random order: (1) a nestmate worker, (2) a non-nestmate worker of the same species (conspecific), (3) a worker of the same genus but a different species (congeneric; *Acromyrmex octospinosus*), (4) a worker of a different genus (*Atta cephalotes*), and (5) a control. All five stimulus types (1-5) were presented in three ways to each ant as either: (a) a dead ant that contacted the antennae, (b) a live ant that did not contact the antennae (presented ~ 10 mm away from the focal ant; held in forceps but not otherwise immobilised), or (c) a live ant that contacted the antennae. The different presentation methods therefore, respectively, exposed the focal ants to cuticular hydrocarbons only (dead ant contact), alarm pheromones (live ant but no contact; the ants generally gaped their mandibles, indicating the release of alarm pheromone), or both (live ant contact), from the stimulus ant. Dead ants were killed by freezing and then defrosted immediately before use in the assays. Both live and dead stimulus ants were medium-sized and medium-aged workers collected from near the entrance to the fungus chamber. The control was a clean metal ball bearing, washed in hexane, then rinsed in water and allowed to dry between each trial. A blind trial was also carried out with 20 ants from two *A. echinator* colonies (40 in total) with three of the treatment ants: a nestmate, a non-nestmate of the same species and a non-nestmate of a different species as well as the same control stimulus used in previous experiments, to confirm that the results were not subject to confirmation bias of the observer (van Wilgenburg and Elgar 2013). Forceps used to present the stimuli were rinsed in hexane and allowed to dry between each presentation.

4.3.3 Experiment 2: the effect of age on threat response behaviour

To look at the effects of age on the threat response, ants of the same size class (1.2 - 1.8 mm head width) but of three different age classes (young, medium and old) were chosen based on their cuticular colour. Six ants of each age class were chosen from each of the six colonies, giving 108 ants in total. Each ant was photographed dorsally using a digital SLR with constant camera settings and lighting conditions. Images were imported into ImageJ software (Schneider et al. 2012) and converted to grayscale, giving a reading of 0 (pure black) to 256 (pure white). Cuticular colour was quantified using the mean value of the middle third of the femur of one of the rear legs, as in Armitage and Boomsma (2010). Mean \pm standard deviation colour for the three age categories were: young (0-20days) 155 ± 12.5 , medium (20-40 days) 133 ± 8.2 and old (40+ days) 108 ± 8.6 . The MOR was tested as described for Experiment 1. The treatments were: a control metal ball, a freshly killed nestmate and a freshly killed *A. cephalotes* worker, which contacted the focal ant's antennae.

4.3.4 Experiment 3: the effect of size on threat response behaviour

To determine how size affects threat response behaviour we used ants of different sizes but the same age. We investigated small (< 1.2 mm), medium (1.2-1.8 mm) and large (> 1.8 mm) size classes (Waddington and Hughes 2010). All ants used for this experiment were of a medium cuticular colour (and therefore of similar age). Six ants of each size class were collected from each of the six colonies, with each ant being dorsally photographed so that size could be quantified by measuring width between the eyes using ImageJ software, a commonly used index of size in ants (e.g. Huang, 2010; Wilson, 1983). Mean \pm standard deviation head widths were: small 1.09 ± 0.085 mm, medium 1.53 ± 0.158 mm and large 1.92 ± 0.099 mm. The MOR of the ants to antennal

contact with a control metal ball, a freshly killed nestmate and a freshly killed *A. cephalotes* worker, were tested as before.

4.3.5 Experiment 4: the effect of experience on threat response behaviour

To determine whether the threat response of ants increased with their experience of stimuli, workers were repeatedly tested once every hour for 6 h on 2 consecutive days, giving 12 repeated trials in total. All ants used in this experiment were of a medium cuticular colour and in the small size range (< 1.2 mm head width). Eighteen ants were taken from each of the six colonies and split into three equal groups. This gave 36 ants for each treatment group. All colonies had no previous experience with non-nestmates. Each group received one of three treatments: a metal ball control, a freshly killed nestmate and a freshly killed *A. octospinosus* worker. This species was chosen rather than *A. cephalotes* because it stimulated lower threat responses in preliminary trials and were thus a better stimulus for detecting the hypothesised increase in the MOR with experience. Harnessed ants were hand-fed a 10 % sucrose solution between days one and two to ensure they were not starved during the trial period.

4.3.6 Statistical analyses

The responses of individual ants in Experiments 1 and 2 were analysed using generalised linear mixed models (GLMM) with binomial distributions and log link functions. The analysis for Experiment 1 examined the effects of treatment (foreignness of the presented ant), method of presentation of the ant (method one, two or three described above), and caste (forager, nest-worker). The analyses for Experiments 2 and 3 examined the effects of treatment (relatedness of the presented ant), and either age (young, medium and old: Experiment 2) or size (small, medium and large: Experiment

3). Colony identity was included as a random factor in all GLMMs. For Experiment 4, we instead analysed the effects of treatment and experience on the total numbers of ants from each colony that showed a MOR over the twelve-time period (i.e., with colonies as replicates rather than a random factor), using a repeated measures general linear model. A Geenhouse-Geisser correction was used to control for deviations from the assumption of sphericity. In all models, non-significant interaction terms were removed in a step-wise manner, using AIC values, to give minimum adequate models. Post hoc pairwise comparisons of treatments used the sequential Bonferroni method to control for multiple comparisons. All statistics were performed in SPSS (v.20 SPSS Inc., Chicago, IL, USA).

4.4 Results

4.4.1 Experiment 1: does threat response behaviour differ between within-nest workers and foragers?

We found a significant difference in MOR between foragers and within-nest leaf-cutting ant workers ($F_{1, 1184} = 45.9$, $P < 0.001$). Foragers showed the MOR more often than within-nest workers in all cases, regardless of the stimulus or presentation method (Figure 4.2). There was a significant interaction between the effects on MOR frequency of stimulus type (nestmate, conspecific, congeneric ant, different genus, or control), and the method of presentation (dead ant contact, live ant contact, or live ant no contact; $F_{8, 1184} = 4.97$, $P < 0.001$). When the stimuli contacted their antennae, the proportions of ants showed a MOR was, in general, greatest to ants of a different genus or congenetics, and least to nestmates, with the conspecifics and control treatments stimulating intermediate MOR frequencies (Figure 4.2a, c). This was not the case when the stimuli

did not contact their antennae, with responses in this case being low regardless of treatment (Figure 4.2b). There was no significant difference between blind and non-blind trials ($F_{1, 279} = 0.148$, $P = 0.701$; Figure S4.1), confirming that the effect seen was not an artefact of observer bias.

4.4.2 Experiment 2: the effects of age on threat response behaviour

Both age and treatment significantly affected the MOR of ants ($F_{2,319} = 7.03$, $P = 0.001$, and $F_{2,319} = 39.10$, $P < 0.001$, respectively). Medium and old workers more frequently showed the threat response behaviour than did young workers (Figure 4.3). For all ages of ants, the MOR was stimulated significantly more frequently by ants of a different genus than by the other stimuli, with nestmates only rarely stimulating a response (Figure 4.3).

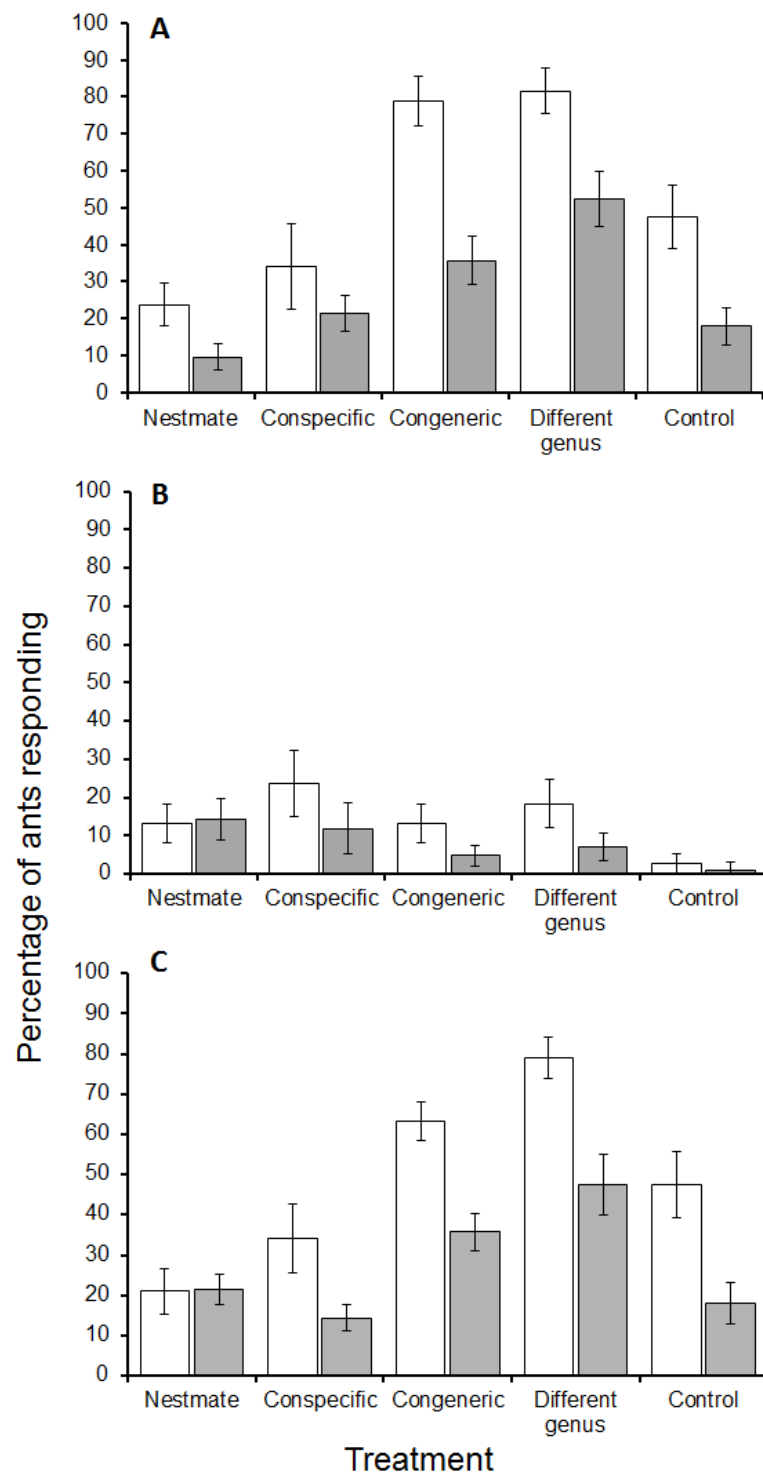


Figure 4.2 MOR data. The mean \pm SE percentage of *Acromyrmex echinator* leaf-cutting ant foragers (white columns) and nest workers (grey columns) from eight colonies that showed a mandible opening response towards stimuli ants of different foreignness to the focal ant, or a control. Stimuli ants were presented as either: (A) a dead ant (or control) that contacted the antennae, (B) a live ant (or control) that did not contact the antennae, or (C) a live ant (or control) that contacted the antennae. The different presentation methods thus exposed the focal ants respectively to the cuticular hydrocarbons, alarm pheromones, or both, from the stimulus ant.

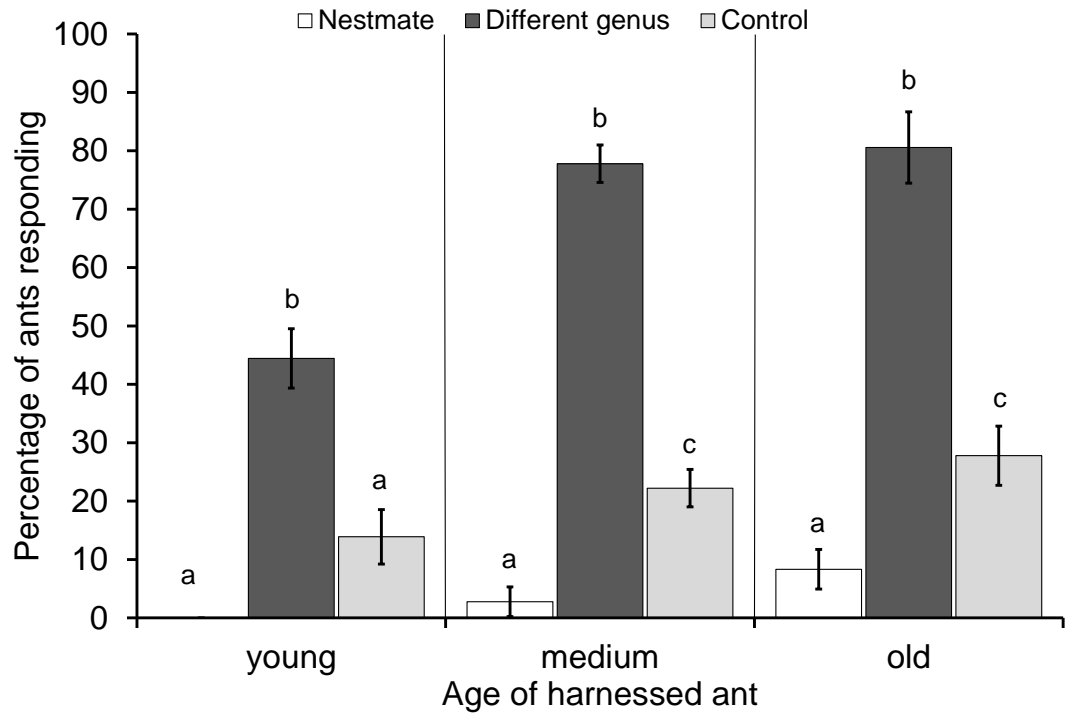


Figure 4.3 MOR age data. The mean \pm SE percentage of young, medium-age and old *Acromyrmex echinator* leaf-cutting ant workers from six colonies that showed a mandible opening response to a nestmate (white columns), an ant of a different genus (dark grey) or a control (light grey columns). Letters represent significant differences within each age category ($P < 0.05$). Stimuli ants were presented as dead ants that contacted the antennae.

4.4.3 Experiment 3: the effect of size on threat response behaviour

Size did not significantly affect the response of the ants to the stimuli ($F_{2, 319} = 0.48$, $P = 0.358$; Figure 4.4). The response to ants of a different genus was extremely similar for small, medium and large workers, and the response to the control stimulus was only moderately higher for small than large workers. Including an ‘extra-large’ category (ants with head widths > 2 mm) for the analysis also did not reveal a significant effect on the response ($F_{3, 320} = 1.061$, $P = 0.304$).

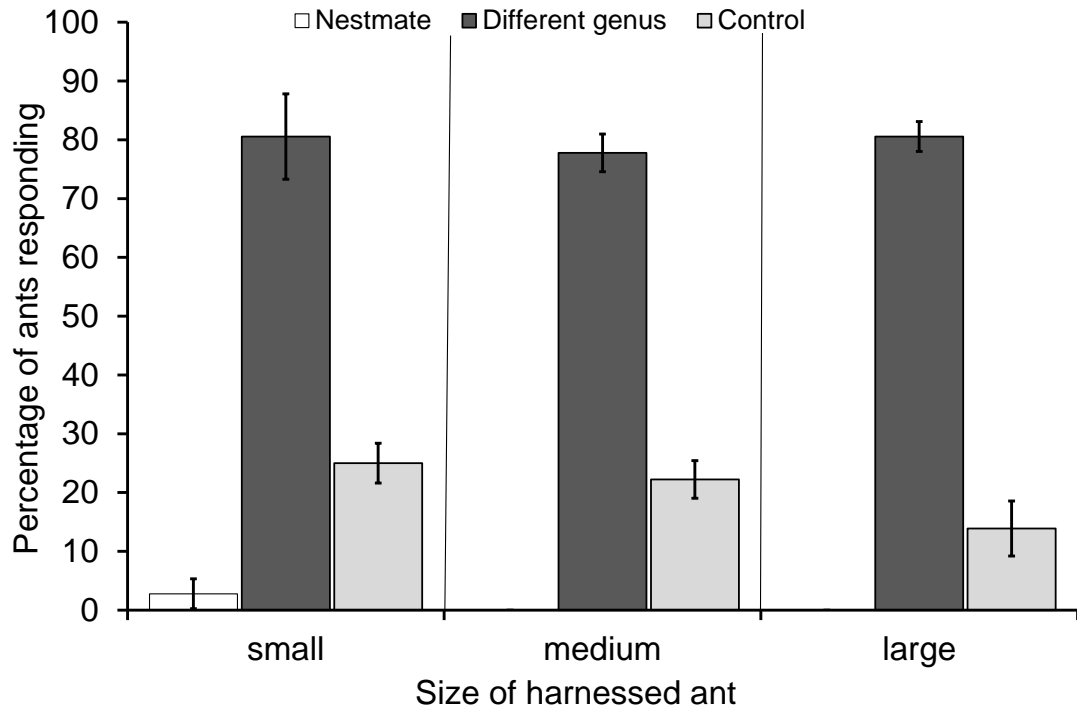


Figure 4.4 MOR size data. The mean \pm SE percentage of small, medium and large *Acromyrmex echinator* leaf-cutting ant workers from six colonies that showed a mandible opening response to a nestmate (white columns), an ant of a different genus (dark grey) or a control (light grey columns).

Stimuli ants were presented as dead ants that contacted the antennae.

4.4.4 Experiment 4: the effect of experience on threat response behaviour

Experience increased the response of the ants with there being a significant interaction between treatment and time ($F_{8, 62} = 6.82$, $P < 0.001$). Both the control stimulus and ants of a different species stimulated significantly more frequent responses than nestmate ants (Figure 4.5), with the responses to the controls and ants of a different species increasing substantially over time. The response of ants to nestmates, in contrast, stayed relatively constant over the repeated exposures, with no more than 10% of ants responding at any point. Responses to ants of a different species and to the control treatment did not differ significantly from one another and reached a plateau after seven exposures (Figure 4.5).

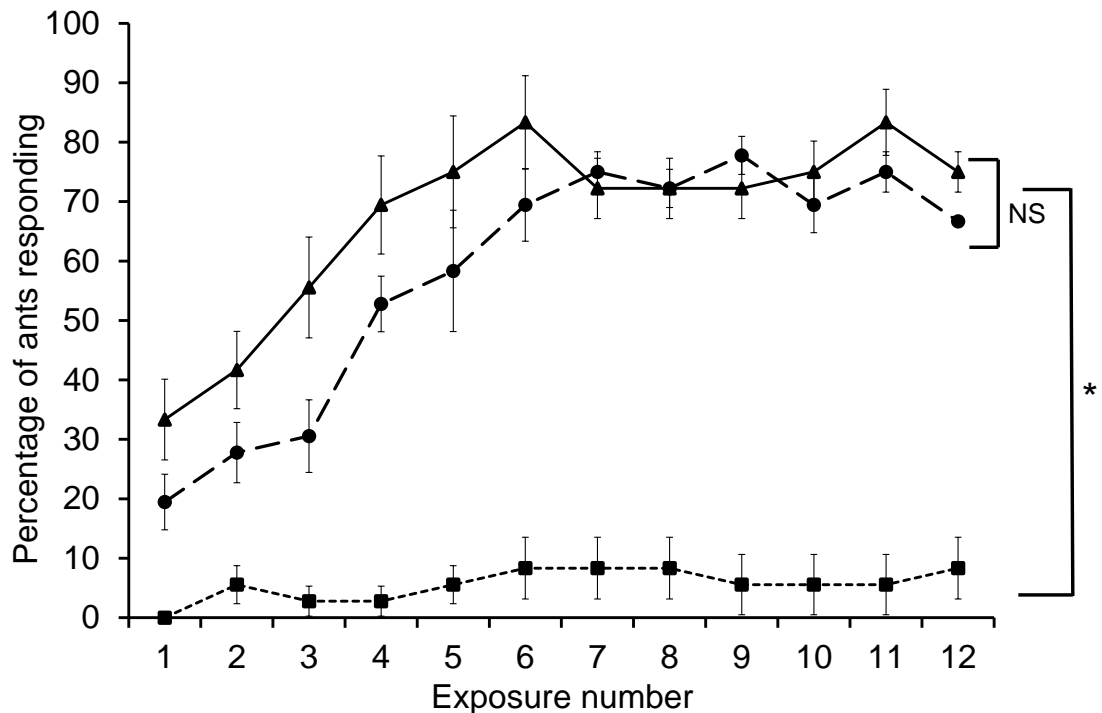


Figure 4.5 MOR experience data. The mean \pm SE percentage of leaf-cutting ant workers from six colonies that showed a mandible opening response to either a nestmate (squares), an ant of a different species (triangles) or a control (circles) in 12 encounters repeated over two days. Stars represent significant differences between stimuli ($P < 0.05$). Stimuli ants were presented as dead ants that contacted the antennae.

4.5 Discussion

Not much is known about the factors influencing threat response behaviour, which is surprising given the large literature on nestmate recognition. We show that individual workers differ strongly in their propensity to display the MOR to threats. Foraging workers responded more readily compared to within-nest workers. Age and experience both positively, and independently, correlate with threat response behaviour.

Surprisingly, we show that size did not predict an individual's propensity to show this behaviour. All ants gave increased responses to increasingly foreign stimuli ants, which was also the case in a blind trial and therefore not due to observer bias (van Wilgenburg and Elgar 2013). Although the foreign control stimuli used stimulated relatively high responses themselves on occasion, the responses to the most foreign ants were

consistently greater. Interestingly, experience had a strong effect on the strength of the response to foreign ants compared to nestmates. These results suggest that the controlled setting of the MOR assay is ideal to test hypotheses on intercorrelating factors affecting an individual's propensity to exhibit threat response behaviour. This assay would also be a useful tool for the study on insect behaviour in general as it could be transferred to any insect which can show behaviours using their mouthparts.

In *Atta* leaf-cutting ants, the composition of the alarm pheromone can differ between colonies (Francelino et al., 2006; Hughes et al., 2001), and ants have been shown to distinguish between nestmate and non-nestmate alarm pheromone in some assays (Whitehouse and Jaffe 1995; Hernández et al. 2002; Francelino et al. 2008). However, we find here that *Acromyrmex* leaf-cutting ants display significantly greater MOR to treatment ants when they contact their antennae than to volatile cues alone. This could suggest that ants use cues from cuticular hydrocarbons more readily than alarm pheromone in the context in this assay, or that any levels of alarm pheromone produced, even by nestmates, were not sufficient to elicit a response here (Wagner et al. 2000; Ozaki et al. 2005; Guerrieri et al. 2009).

Threat response behaviour increased with the foreignness of the focal ant to the treatment ant meaning ants can apportion their threat response according to how similar the intruder is to the receiver. This is most likely because the cuticular hydrocarbon profile of more foreign ants differs more from the responding ant's nestmate template, as found by Guerrieri and D'Ettorre (2008). Ants were always least responsive to nestmates, suggesting that being in a harness did not impair their ability to distinguish nestmate from non-nestmate. What is surprising, however, is the lack of significant discrimination between nestmates and individuals of the same species, given the importance of nestmate recognition in leaf-cutting ants and social insects more

generally (Hamilton 1964; Hölldobler and Wilson 1990; Hernández et al. 2002; Hernández et al. 2006). Laboratory colonies of ants are known to be less aggressive to conspecifics than field colonies (Obin and Vander Meer 1988; Crosland 1989). Different food sources are also known to modify cuticular hydrocarbons, and subsequent nestmate recognition, in *Acromyrmex subterraneus subterraneus* and *A. octospinosus* leaf-cutting ants, as well as the Argentine ant *Linepithema humile* and *Camponotus herculeanus* (Jutsum et al. 1979; Liang and Silverman 2000; Richard et al. 2004; Guerrieri et al. 2009). All laboratory colonies were reared on the same diet, perhaps leading to smaller differences in cuticular hydrocarbons between colonies than found in natural settings.

Surprisingly, given the role of larger individuals in nest defence (Wilson 1983; Nowbahari et al. 1999; Huang 2010; Grüter et al. 2012), size was not found to affect the response of ants to threats. However, the MOR assay only tests threat response behaviour in general, not specifically aggression. All sizes of individuals recognise conspecifics in equal amounts but it is still possible that larger individuals may react more aggressively following threat recognition. In *Atta* species, the larger, soldier caste responds more readily in response to vertebrate predation, although they are unable to distinguish conspecifics from separate nests (Salzemann and Jaffe, 1991; Wilson, 1980), whereas smaller ants may be recruited more readily in response to a conspecific threat (Whitehouse and Jaffe 1996; Hughes and Goulson 2001; Hölldobler and Wilson 2010).

In contrast, age did affect threat response behaviour, with young ants less frequently showing a MOR to all treatments compared to medium and old-aged ants. This fits in with other nestmate recognition studies which found that younger individuals, especially callows, require a learning period to develop the nestmate template that they need to compare all other chemical templates to (Gamboa et al.,

1986; Jaisson, 1991; Sturgis and Gordon, 2012a). Many social insects, leaf-cutting ants included, also exhibit age-based polyethism in which older workers carry out more dangerous, external tasks (Wakano 1998; Julian and Fewell 2004; Camargo et al. 2007; Waddington and Hughes 2010), that may make recognising threats from non-nestmates potentially more important. The effect of age may therefore be a physiological constraint and also adaptive.

What is most interesting, however, is that the effect of experience (independent of age) also showed an increase in threat response behaviour. Experience is known to affect behaviour expression in social insects, for example, its effects on foraging are particularly studied (Beshers and Fewell 2001; Robinson et al. 2012; Hagbery and Nieh 2012). Artificially created experience over the experimental time period in medium-aged ants caused an increase in threat response behaviour towards both the congeneric ant and control treatment but not the nestmate treatment. This is suggestive not of associative learning by the focal workers but of sensitization learning, allowing a rapid induction of a threat response behaviour to an immediate threat (van Wilgenburg et al. 2010; Rittschof and Robinson 2013b). Although the fact we only observe such an increase in focal ants repeatedly shown a non-nestmate, responses to the control ball and a nestmate is most interesting. The exact cause behind this is speculative. Some studies have found that experience of a non-nestmate lowers the response threshold for aggression (van Wilgenburg et al. 2010), while others have reported a raising of the response threshold to non-nestmates due to habituation (Stroeymeyt et al. 2010). Our results agree with the former, with an increase in threat response behaviour during the experiment and no evidence of habituation. It is worth noting that in the context of other aspects of this experiment experience is not the only factor affecting an individual in their ability to show an appropriate threat response.

The fact that age and experience act separately indicates a more complex expression of these types of behaviours than previously thought, with both factors seeming to have separate underlying mechanisms. It is likely that lowering of the threshold is coupled with other mechanisms, such as potential physiological effects of age which may explain why certain individuals tend to react more strongly to non-nestmates. The results suggest that experience may be a crucial factor in nestmate recognition and highlight the complexity of factors contributing to the expression of behaviours; where those that appear to be similar can act as separate entities. The importance of assessing the individual contribution of effects that act synergistically will be particularly useful in assessing the reasons why individuals differ in their behaviours.

5 Alarm pheromone composition and behavioural activity in fungus-growing ants



Figure 5.1 An *Atta laevigata* soldier shows an alarm response towards a vertebrate intruder (a.k.a. an overly enthusiastic photographer). An *A.laevigata* soldier displays a typical alarm response gaping her mandibles to release alarm pheromone to communicate a threat to her nestmates.

5.1 Abstract

Chemical communication is a dominant method of communication throughout the animal kingdom and can be especially important in group-living animals in which communicating threats, either from predation or other dangers can have large impacts on group survival. Social insects in particular have evolved a number of pheromonal compounds specifically to signal alarm. There is predicted to be little selection for interspecific variation in alarm cues because individuals may benefit from recognizing interspecific, as well as conspecific, cues, and consequently alarm cues are not normally thought to be used for species or nestmate recognition. Here we examine the composition of the alarm pheromones of seven species of fungus-growing ants (Attini), including both basal and derived species, and examine the behavioural response to alarm pheromone of *Acromyrmex* leaf-cutting ants, the sister genus to the highly studied *Atta* leaf-cutting ants. We find surprisingly high interspecific variation in alarm pheromone composition across the attine phylogeny. Interestingly, the behaviourally active component of the alarm pheromone was different even between the two leaf-cutting ant genera. Furthermore, in contrast to previous studies on *Atta*, we found no differences between morphological castes in their behavioural responses to alarm pheromone in *Acromyrmex*, but we did find age-related differences in behavioural responses. The results suggest that the evolution of alarm communication and signalling within social insect clades can be unexpectedly complex and that further work is warranted to understand whether the evolution of different alarm pheromone compounds is adaptive with regards to communication.

5.2 Introduction

Alarm communication is shown by a wide variety of organisms including vertebrates, invertebrates and even plants (Chivers and Smith 1998; Heil and Ton 2008; Shah 2009; Wyatt 2014). However, alarm communication is particularly beneficial when individuals live in groups because the rapid communication of threats to group members enables groups to form collective responses to stimuli (Nault and Phelan 1984; Verheggen et al. 2010). In many organisms these alarm signals are visual (Murphy 2006; le Roux et al. 2008), but in insects they are predominantly chemical, with alarm pheromones being the secondly most commonly produced class of compounds (Regnier and Law 1968; Verheggen et al. 2010).

Alarm communication is particularly diverse in the eusocial insects such as ants, in which a wide variety of compounds function as stimuli for alarm behaviours (Bowers et al. 1972; Crewe et al. 1972; Cammaerts et al. 1983; Verheggen et al. 2010; Bortolotti and Costa 2014). These allow individuals within a colony to respond rapidly, and appropriately, to an alarm stimulus. There are two main behavioural responses to alarm cues which serve separate functions (Wilson and Regnier 1971; Billen and Morgan 1998; Verheggen et al. 2010). The first is a panic response in which responders show escape or flight behaviours to disperse from the threat. The second is an aggressive response in which workers are attracted to, and attack, the threat. These responses can depend upon a variety of factors, including concentration of pheromone, compounds within the pheromone, colony size, and the spatial context of the communication (Shorey 1973; Kerr et al. 1973; Topoff et al. 1989; Hölldobler and Wilson 1990; Vander Meer and Alonso 1998; Hughes and Goulson 2001).

We can also expect differences between individual workers in their response to alarm cues. Polyethism in complex eusocial insect societies can be based on age (temporal polyethism) or morphological phenotype (caste). Many insects show a

specialised defensive caste, be it guards in honey bee colonies, or morphologically specialised soldiers in stingless bee, ant, termite, aphid or thrip colonies (Boch et al. 1962; Stern and Foster 1996; Shibao 1998; Hölldobler and Wilson 2010; Grüter et al. 2012). These individuals are often the first line of defence for the group and respond more aggressively to defensive stimuli (Whitehouse and Jaffe 1996; Nowbahari et al. 1999; Hölldobler and Wilson 2009; Alaux et al. 2009). There is variation between colonies in alarm responses as well as between species, for example the much greater defensiveness of Africanized honey bees compared to their European counterparts (Collins et al. 1982; Giray et al. 2000; Schneider et al. 2004). However, the reduced need for specificity in alarm pheromones compared to other pheromones means that there is predicted to be relatively little selection for the evolution of species-specific alarm pheromones (Blum 1969; Vander Meer and Alonso 1998). Indeed, in other organisms such as fish or crustaceans a lack of species specificity can be an advantage in alarm cues (Hazlett 1994; Commens and Mathis 1999; Laforsch et al. 2006).

The tribe Attini provides an ideal system for testing this hypothesis within the social insects. This clade exhibits varying levels of social complexity. Leaf-cutting ants (*Atta* and *Acromyrmex*) are the most derived clade within the fungus-growing ants (tribe Attini), all of which are characterised by culturing a mutualistic fungal crop. The two ecologically dominant leaf-cutting ant genera are distinguished from other attines by cultivating their fungal crop generally on fresh vegetation, and having much larger and more complex societies, with thousands to millions of polymorphic workers compared to tens to hundreds of monomorphic workers in the basal attines (Weber 1972). The higher leaf-cutting genera of *Acromyrmex* and *Atta* show some of the most complex forms of division of labour with extreme caste polymorphism in which workers can vary in size by as much as six-fold (Wilson 1980; Hölldobler and Wilson 2010). Within

leaf-cutting ant colonies, smaller workers tend to carry out internal work within the nest, such as caring for the brood and fungus garden, while larger workers tend to carry out the foraging and nest defence, and soldiers (only present in *Atta*) specialise normally in nest defence (Wilson 1980; Wetterer 1995). As with many other ant and bee species, the alarm pheromone in leaf-cutting ants is produced by the mandibular glands and is released when the mandibles are gaped in response to an alarm stimulus (Blum 1969; Bradshaw et al. 1975; Verheggen et al. 2010). The composition of the alarm pheromone in *Atta* has been relatively well studied and all six of the species so far investigated (*A. bisphaerica*, *A. capiguara*, *A. laevigata*, *A. opaciceps* and *A. sexdens*) have 4-methyl-3-heptanone as the predominant and most behaviourally active component (Blum et al. 1968; Moser et al. 1968; Crewe and Blum 1972; Do Nascimento et al. 1993; Hernandez et al. 1999; Hughes et al. 2001a; Francelino et al. 2006). Depending on the species and caste, the alarm pheromone of *Atta* can also contain a diversity of other volatile compounds, with as many as 41 volatile compounds being found in head extracts (Hughes et al. 2001a), but the roles of these other compounds is unclear. There is also clear evidence of polymorphism in the composition, activity and receptiveness of *Atta* ants to alarm pheromone. While large workers and particularly soldiers tend to produce relatively complex pheromonal mixtures, the alarm pheromone of small workers (minims) in *Atta* is simpler, consisting predominantly of 4-methyl-3-heptanone (Do Nascimento et al. 1993; Hernandez et al. 1999; Hughes et al. 2001a). This implies that the small workers in *Atta* may produce a particularly potent alarm pheromone in terms of composition, though the quantities they produce are lower than their larger nestmates given their smaller mandibular glands (Hernández and Caetano 1995). Although all castes respond to alarm pheromone (Wilson 1980), field studies have found that smaller workers, although less abundant on foraging trails, are disproportionately abundant in

the ants responding to alarm stimuli, suggesting that they may play a key role in detecting and responding to threats (Hughes and Goulson 2001).

The rich literature on the composition and behavioural activity of alarm pheromone in *Atta* leaf-cutting ants contrasts markedly with the very limited investigation of alarm pheromones in other fungus-growing ants. Only two studies have investigated the chemical composition of the mandibular gland pheromones in other Attini, and these suggest intergeneric differences, with eight *Trachymyrmex* species appearing to lack 4-methyl-3-heptanone (Crewe and Blum 1972; Adams et al. 2012). These results are therefore intriguing because they suggest that different fungus-growing ant taxa may have evolved different alarm pheromone compounds, in contrast to the predicted lack of selection for interspecific variation in alarm pheromone. However, detailed analyses of the composition of alarm pheromones, and controlled assays of behavioural activity, are needed for *Acromyrmex* and other attines to resolve this. Here, we identify and compare the chemical composition of the alarm pheromone for two species of *Atta* that have not previously been investigated (*At. colombica* and *At. cephalotes*), two species of *Acromyrmex* (*Ac. echinator* and *Ac. octospinosus*), two other ‘higher’ attines (*Sericomyrmex amabilis* and *Trachymyrmex cornetzi*), and the ‘lower’ attine *Apterostigma pilosum* (a coral fungus ant, representing the most basal of the attines). We also examine the behavioural activity of the most abundant components of the alarm pheromones for the two *Acromyrmex* species, comparing this with the behavioural responses of the two *Atta* species to their most behaviourally active components, and finally examine how the caste and age of *Acromyrmex* workers affects their responsiveness. We do this using a precise, individual-level assay based on the mandible opening response (Guerrieri and D’Ettorre 2008), a response which is stimulated in ants by alarm pheromones regardless of whether they would exhibit a

panic or aggressive response (Grover et al., 2007; Hölldobler and Wilson, 1990; Hughes et al., 2001; Norman et al., 2014).

5.3 Methods

5.3.1 Chemical composition of alarm pheromone in Attine ants

Workers were collected from colonies of *Apterostigma pilosum*, *Sericomyrmex amabilis*, *Trachymyrmex cornetzi*, *Acromyrmex echinator*, *Acromyrmex octospinosus*, *Atta cephalotes* and *Atta colombica* that had been collected from Gamboa, Panama in May 2013 and maintained in the laboratory for 6-18 months. We collected 20 workers from each of 2-4 colonies of the monomorphic *Ap. pilosum* (3 colonies), *T. cornetzi* (2 colonies) and *S. amabilis* (4 colonies). For the polymorphic leaf-cutting ants, we collected 20 small (<1.5 mm head width) and 20 large workers (2.0-3.0 mm head width) from 2-4 colonies of *Ac. echinator* (4 colonies), *Ac. octospinosus* (3 colonies), *At. colombica* (2 colonies) and *At. cephalotes* (2 colonies). Ants were immediately cooled on ice after collection, their heads removed and placed into 100 µl of hexane (97% pure) containing 10ng/ant head of an internal standard (5-methyl-3-heptanone 97%, Sigma Aldrich). Heads were crushed thoroughly with a clean glass rod, vortexed for 60 s, placed in a sonic bath for 60 s, spun down for 60 s, filtered through a glass Pasteur pipette plugged with clean glass wool, and the extract was stored at -20°C until analysis. Whole crushed heads were used as previous studies on multiple genera of leaf-cutting ants have found no difference in the volatiles identified when comparing extracted mandibular glands (the source of the alarm pheromone) to whole crushed heads (Blum et al. 1968; Moser et al. 1968; Do Nascimento et al. 1993; Murakami et al. 2000; Hernández et al. 2002; Francelino et al. 2008).

The sample extracts were analysed by gas chromatography–mass spectrometry (GC-MS), using a Perkin Elmer instrument consisting of an Autosystem XL GC and TurboMass MS. The column used was a Supelco SLB-5ms, 30 m x 0.25 mm ID x 0.25 μ m film thickness, (Sigma Aldrich). The temperature program was 40°C (hold for 3 min), increasing at 10°C/min to 75°C, increasing at 20°C/min ending with 300°C for 5 min. Samples were injected using a splitless injection with a splitless time of 1.0 min; the inlet temperature was 250°C and injection volume 0.3 μ L. The carrier gas was Helium with a flow rate of 1.3 ml/min. The instrument was operated in full scan mode with a mass range of m/z 20-650. Transfer line and MS source temperatures were 300°C and 250°C respectively. Compounds were identified by searching a database (NIST MSSearch 2.0) for matching mass spectra then confirmed by comparing retention times and mass spectra with known standards.

5.3.1.1 Experiment 1: Behavioural activity of compounds in leaf-cutting ants

In order to determine the behaviourally active components of the alarm pheromone, the mandible opening response (MOR) assay was used (Guerrieri and D'Ettorre 2008; Norman et al. 2014). This individual-level assay is ideal because individual ants can be isolated and their threat response behaviour towards specific chemical stimuli under controlled conditions easily ascertained by whether or not the focal ant gapes its mandibles after exposure to the stimulus (indicating recognition of a threat, and therefore capturing both panic and aggression responses: Grover et al., 2007; Hölldobler and Wilson, 1990; Hughes et al., 2001; Norman et al., 2014).

To carry out the MOR assay, ants were chilled on ice until immobilised and then harnessed in 0.2 ml pipette tips (Starlab, Bucks, UK), cut at the apex through which the head of the ant was passed and secured with a thin strip of masking tape. Ants were left for 2 h in the harness to acclimatise before being assayed, and for at least 30 min

between chemical stimuli to avoid habituation. In order to test which of the chemicals identified in the alarm pheromone extract were behaviourally active, compounds identified as consistently present in the extracts of either *Atta* or *Acromymex* species were tested on their respective species using the mandible opening response (MOR) described above. Neat compounds were used for this assay as preliminary data showed these caused the highest levels of response (V. C. N., unpublished data). We compared the response of 30 large workers (2.0 – 2.5 mm head width) and 30 small workers (1.0-1.5 mm head width) per species, sampled equally from five colonies of *Ac. echinator*, *Ac. octospinosus* and *At. colombica*, or from the single available colony of *At. cephalotes*. To simulate the volatile emission of alarm pheromone in nature, a 20 μ l drop of neat compound was placed on filter paper 10 mm from the antennae of the focal ant and the response of the ant was recorded. A 20 μ l drop of water applied in the same way was used as a negative control, and the crushed head of a freshly freeze-killed nestmate used as a positive control.

5.3.1.2 Experiment 2: The effect of caste and age on alarm response in *Acromymex*

To determine if the caste or age of *Ac. echinator* and *Ac. octospinosus* leaf-cutting ant workers affected their response to alarm pheromone, we again utilised the MOR assay. To examine the effect of caste, we compared the responses of 30 small workers, 30 medium workers, and 30 large workers (1.25 ± 0.03 , 1.65 ± 0.02 , and 2.09 ± 0.03 mm head widths respectively, sampled equally from five colonies for each species). All ants were of a medium cuticular colouration, indicating similar ages (Armitage and Boomsma 2010). This was confirmed by quantifying the cuticular colouration of a subset of 30 per caste from a dorsal photograph taken with a Canon EOS 350d dSLR camera and Canon EF 100 mm f/2.8 Macro lens under constant lighting. Images were imported into ImageJ software and converted to grayscale, giving a reading of 0 (pure

black) to 256 (pure white). Cuticular colour was quantified using the mean value of the middle third of the femur of one of the rear legs, as in Armitage and Boomsma (2010). To examine the effect of age on the threat response, ants were selected of similar size (1.2-1.8-mm head width) but of three different age classes based on their cuticular colouration (young, medium and old). Six ants of each age class were chosen from each of the five colonies for each species, giving 90 ants in total per species. To confirm their ages, each ant was photographed and cuticular colouration quantified as described above. Mean \pm s.e. colour for the three age categories was: young (0–20 days) 93.4 ± 1.37 , medium (20–40 days) 70.5 ± 0.752 and old (40+ days) 57.6 ± 0.930 . In both the caste and age experiments, we tested the response of ants to the most behaviourally active compound (3-octanol for *Ac. echinator* and 3-octanone for *Ac. octospinosus*), using a dosage of each compound that corresponded to the amount found in a single large worker ant head in order to test a biologically relevant concentration of the compounds (25 ng for *Ac. echinator*, 135 ng for *Ac. octospinosus*). Compounds were dissolved in hexane to 1 ng/ μ l first, then placed on filter paper, left for 15 s to allow the solvent to evaporate, and the filter paper placed within 10 mm of the antennae of the focal ant. Worker's responses to these 'head-realistic' doses were compared to a solvent negative control (prepared in the same way as the head-realistic doses, but with an equivalent volume of hexane only), and a crushed head of a freshly freeze-killed nestmate as a positive control.

5.3.1.3 Experiment 3: Colony-level assay.

To confirm that the MOR assay was appropriate for detecting alarm responses, we also carried out colony-level assays using *Ac. echinator* and *Ac. octospinosus*, both to confirm in a more colony realistic set-up that the ants exhibited alarm behaviours in response to the compounds and that individuals show mandible gaping when alarmed.

For each of the compounds found consistently in the extracts for each species, individual 50 mm filter paper discs containing the equivalent amount of compound found in one crushed ant's head were used as alarm stimuli. Once applied to the disc, the solvent was left to evaporate for 15 s and then the disc placed at least 50 mm away from the nest entrance. Each assay was filmed for 1 min with snapshot behaviours recorded at 10 s intervals during this period. The number of ants gaping mandibles was recorded at each 10 s, as well as the number of ants present on the filter paper to quantify attraction or arrestment. This was carried out for four *Ac. octospinosus* colonies and three *Ac. echinator* colonies. A crushed head of a freshly freeze-killed nestmate was used as a positive control.

5.3.1.4 Experiment 4: Confirmation of behavioural responses to volatiles released by ants.

Finally to confirm that focal ants were reacting to volatiles released by alarmed ants, and not just to compounds found in crushed whole heads, we carried out a further experiment utilising the MOR assay. Focal ants were exposed to five stimuli in a random order. 1) A live alarmed nestmate (with gaping mandibles indicating that the stimulus ant was alarmed and releasing alarm pheromone) held 10 mm away from the head of the focal ant. 2) A dead, nestmate as a control, which could not be releasing alarm pheromone. 3) Eight live nestmates, in a sealed pot (25 mm diameter, 40 mm height), that had been alarmed for 20 s, with the pot then placed 10 mm from the the focal ant and opened. 4) The same treatment but under red light, to ensure focal ants were not reacting to the sight of alarmed ants. 5) An empty pot as a control. These assays were carried out for 40 *Ac. echinator* individuals (sampled evenly from four colonies) and 50 *Ac. octospinosus* individuals (sampled evenly from five colonies).

5.3.2 Statistical Analysis

The programme PRIMER 6, version 6.1.13, was used with the permutational multivariate analysis of variance (*PERMANOVA*) version 1.0.3 for multivariate analysis of percentage composition to determine differences between species of the compounds present in the alarm pheromone. This analysis, a non-parametric *MANOVA*, has the advantage of being free from assumptions on data distribution (Anderson et al. 2008). All multivariate analyses were carried out using 9,999 permutations on a resemblance matrix using Euclidian distance estimates. We used a one-factor *PERMANOVA* design, with species identity as a fixed factor. All peaks identified as present in the alarm pheromone (41 in total over the 7 species) were included in the analysis. A further *PERMANOVA* model was constructed to calculate post-hoc pairwise comparisons between species groups. We also carried out a one-way similarity of percentage analysis (*SIMPER*), to further analyse qualitative differences between species in alarm pheromone composition. This calculates the contributions of specific chemicals to the separation of species by the chemical composition in a non-metric multidimensional scaling analysis (*MDS*). A canonical analysis of principal components (*CAP*) was also carried out in order to predict group membership and help confirm the effectiveness of the functions in discriminating between the groups.

All behavioural data were analysed using generalized linear mixed models (*GLMM*), which included colony-of-origin as a random factor, except for *At. cephalotes* for which only one colony was used and the data therefore analysed with a generalized linear model (*GLM*). For colony-level assays, time point nested within colony was used as a random factor. For colony-level assays, number of ants and number of mandible opening responses (MORs) were analysed using a poisson distribution and a log link function. In all individual-level mandible opening response experiments (MORs) were

compared using a binomial distributions and logit link function, with stimuli in the first experiment, and stimuli and caste or age in the second experiments, as factors. Non-significant interaction terms were removed stepwise in all cases to obtain the minimum adequate models. Sequential Bonferonni corrections were used to adjust for multiple comparisons during pairwise post-hoc testing. All behavioural analyses were performed in SPSS (v.20 SPSS Inc., Chicago, IL, USA).

5.4 Results

5.4.1 Chemical composition of alarm pheromone in Attine ants

Alarm pheromone composition differed significantly between the seven species tested ($F_{6, 27} = 20.3$; $P < 0.001$). All species differed significantly in their compositions from one another except for *Ac. octopsinosus* and *Ac. echinator* ($P = 0.521$), and also *At.cephalotes* and *S. amabilis* ($P = 0.604$). There was little congruence across genera between their phylogeny and the chemical similarity of their alarm pheromone (

Figure 5.2a). This is supported by MDS analysis, which separated the species into three clusters, with the *Acromyrmex* species clustering together, mostly differentiated from other attines by the presence of 3-octanone (

Figure 5.2b). Interestingly, *S. amabilis* clustered with the two *Atta* species, differentiated from the other attines by the presence of 4-methyl-3-heptanone. *T. cornetzi* and *Ap. pilosum* made up the third cluster and were both mostly distinguished

from the other two clusters by the almost complete lack of either 4-methyl-3-heptanone or 3-octanone.

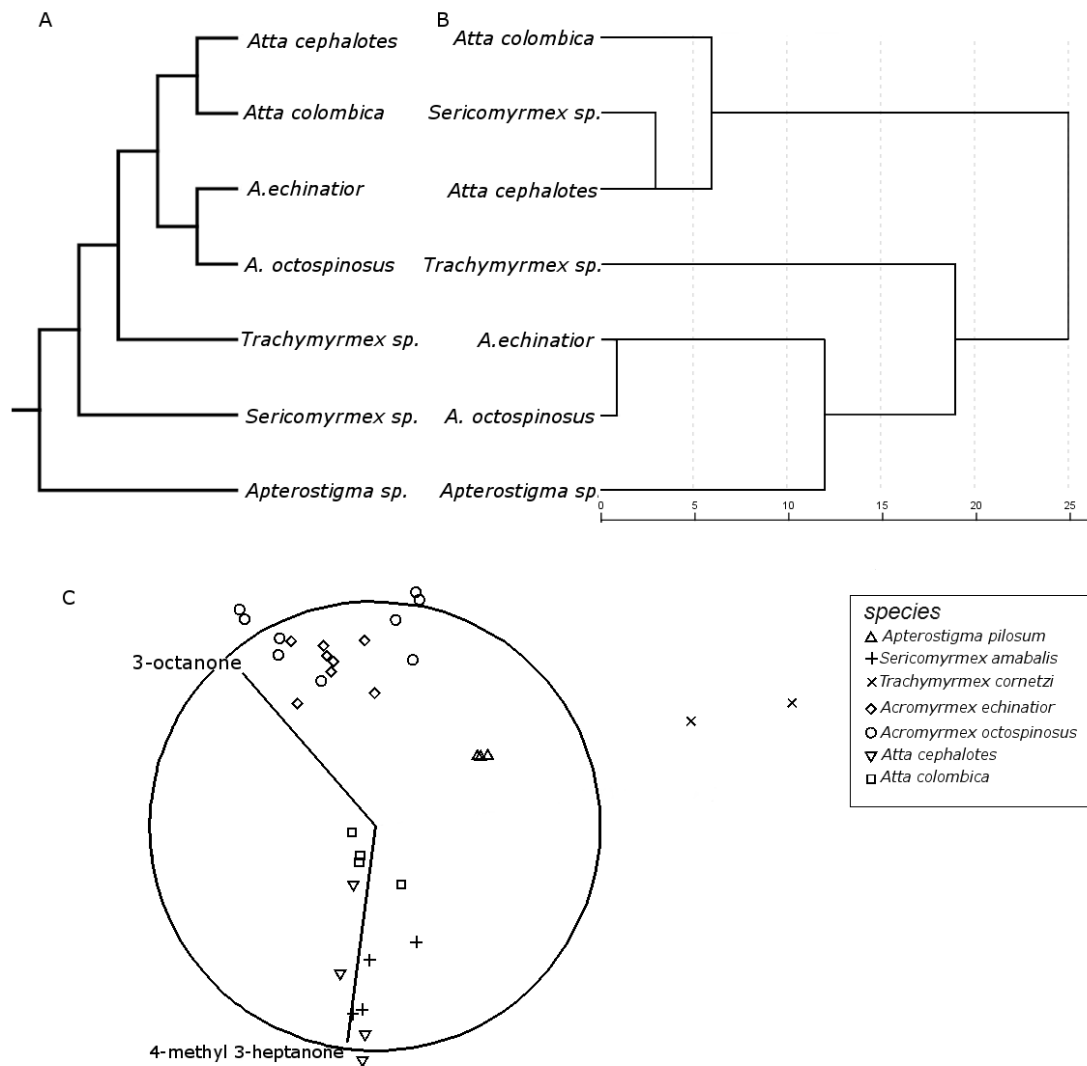


Figure 5.2 Composition of the alarm pheromone of seven attine fungus-growing ant species (A)

Phylogeny of the seven attine species (adapted from Schultz and Brady 2008) and (B) dendrogram showing similarity in the composition of alarm pheromone composition, calculated using Euclidian distances with shorter distances between chemical profiles indicating greater similarity. (Cc) Multi-directional scaling (MDS) plot, showing the similarity of alarm pheromone composition for the seven attine species: *Apterostigma pilosum* (open upright triangles), *Trachymyrmex cornetzi* (rotated crosses), *Sericomyrmex amabilis* (crosses), *Acromyrmex echinator* (open diamonds), *Acromyrmex octospinosus* (open circles), *Atta cephalotes* (open upturned triangles) and *Atta colombica* (open squares) calculated using Euclidian distances. Each symbol is from a sample of 20 workers from one colony, with shorter

distances between symbols indicating greater similarity in pheromone composition. Straight lines indicate the main axis of differentiation between the samples and the main chemicals causing this grouping.

In total, 29 compounds of high volatility occurred consistently in one or more of the species. Of these, 24 compounds could be identified with a high degree of confidence; including all of the most abundant peaks (Table 5.1). The CAP analysis correctly identified the species of 91% of the 34 samples. Misclassification of species only occurred in 3 out of the 7 species, with 25% of samples being misclassified for *At. cephalotes* (1/4 samples), *At. colombica* (1/4) and *S. amabilis* (1/4), in all cases as other species within this cluster.

5.4.1.1 Experiment 1: Behavioural activity of compounds in leaf-cutting ants

All four species of leaf-cutting ants showed significant differences in their mandible opening response (MOR) behaviour to the different compounds tested ($F_{8,530} = 7.99$, $P < 0.001$; $F_{8,530} = 8.93$, $P < 0.001$; $F_{7,471} = 6.62$, $P < 0.001$; $\chi^2_8 = 51.8$, $P < 0.001$ for *Ac. echinator*, *Ac. octospinosus*, *At. colombica* and *At. cephalotes*, respectively; Figure 5.3). For all species, crushed heads caused significantly more positive MORS than any of the single compounds tested, and there were minimal responses to the water negative control. The most behaviourally active compound for both *At. cephalotes* and *At. colombica* was 4-methyl-3-heptanone (Figure 5.3 c & d). The most behaviourally active compound for *Ac. echinator* was 3-octanol, whereas for *Ac. octospinosus* this was 3-octanone (Figure 5.3 a & c). There was no significant difference between large and small workers in their responses to neat compounds for *Ac. echinator*, *Ac. octospinosus* and *At. cephalotes* ($F_{1,530} = 0.204$, $P = 0.653$; $F_{1,530} = 0.315$, $P = 0.989$; $\chi^2_1 = 0.995$, $P = 0.319$ respectively), whereas for *At. colombica* small workers were significantly more threat responsive than large workers ($F_{1,471} = 19.1$, $P < 0.001$; Figure 5.3c).

5.4.1.2 Experiment 2: The effect of caste and age on alarm response in *Acromyrmex*.

Ants in all assays responded most strongly to the crushed head positive controls, followed by the head-realistic doses of the most behavioural active compound, and showed the least response to the negative control (Figure 5.4). For both *Ac. echinator* and *Ac. octospinosus* there was no significant effect of caste on the proportion of ants showing a MOR ($F_{2,265} = 0.019$, $P = 0.981$; $F_{2,265} = 1.81$, $P = 0.166$, respectively; figure 3a-b). However, there was a significant effect of age on the MOR for both *Ac. echinator* and *Ac. octospinosus* ($F_{2,265} = 17.0$, $P < 0.001$; $F_{2,265} = 14.0$, $P < 0.001$, respectively). Older workers were more threat responsive than younger workers, for all stimuli and in both species (Figure 5.4 c-d).

5.4.1.3 Experiment 3: Colony-level assay.

There were significant differences between the treatments in the numbers of ants within 50 mm of the stimulus ($F_{7,136} = 18.6$, $P < 0.001$; $F_{7,184} = 16.8$, $P < 0.001$). The numbers of ants attracted or arrested near the stimulus were significantly higher in response to crushed heads and either 3-octanol (for *Ac. octospinosus*) or 3-octanone (for *Ac. echinator*), than in response to the other compounds or the control (Figure 5.5 a & c). The treatments similarly differed significantly in the numbers of ants exhibiting gaping mandibles following presentation of the stimuli ($F_{7,136} = 23.0$, $P < 0.001$; $F_{7,184} = 54.2$, $P < 0.001$ for *Ac. echinator* and *Ac. octospinosus* respectively), with significantly more ants gaping mandibles in response to crushed heads and 3-octanol or 3-octanone (depending on species) than to the other compounds or control (Figure 5.5).

Table 5.1: Volatile compounds found in the mandibular glands of seven attine fungus-growing ant species. The most abundant compound for each species is highlighted in bold and the most behaviourally active underlined.

Compound	<i>Apterostigma pilosum</i>	<i>Sericomyrmex amabilis</i>	<i>Trachymyrmex cornetzi</i>	Volatiles (% mean \pm s.e)		<i>Acromyrmex echinator</i>	<i>Atta colombica</i>	<i>Atta cephalotes</i>			
				LW	SW			LW	SW		
2-ethyl-1-hexanol	0	4.39 \pm 1.56	0	4.23 \pm 3.33	2.05	5.98 \pm 2.02	1.48 \pm 3.99	19.4 \pm 2.9	17.6 \pm 8.8	19.81	10.8 \pm 10.8
3-octanol	0	0	4.1 \pm 0.9	4 \pm 2.23	2.9	<u>6.22 \pm 2.39</u>	<u>5.0 \pm 2.71</u>	0	14.2 \pm 7.1	0	0
3-octanone	2.04 \pm 0.286	0	0	<u>38.5 \pm 13.5</u>	<u>41.12</u>	<u>40.6 \pm 1.06</u>	<u>42.0 \pm 1.70</u>	2.47 \pm 1.8	16.2 \pm 8.1	0	0.933 \pm 0.93
4-methyl-3-heptanone	0	70.7 \pm 5.34	0	0	0	0	0	<u>41.7 \pm 2.3</u>	<u>41.6 \pm 2.9</u>	<u>69.7</u>	<u>72.0 \pm 14.6</u>
2-nonanone	0	0	0	3.33 \pm 2.06	5.18	6.38 \pm 2.46	7.4 \pm 2.86	0	0	0	0
2-undecanone	0	0	0	28.9 \pm 8.96	36.97	24 \pm 1.31	33.5 \pm 1.48	0	1.61 \pm 0.8	0	0
nonanal	0	0	0	0	0	0.725 \pm 3.32	0	0	0	0	0
nonane	0	0	0	4.93 \pm 1.09	1.367	13.8 \pm 1.21	4.0 \pm 2.41	6.7 \pm 0.6	2.5 \pm 0.28	5.32	1.02 \pm 1.02
4-methyl-3-heptanol	0	8.4 \pm 7.36	1.7 \pm 1.7	0	0	0	0	4.8 \pm 0.2	9.97 \pm 6.7	0	5.04 \pm 2.96
octanal	0	1.31 \pm 0.736	0	0.567 \pm 0.57	0	0	0	0	0	0	0
2-heptanone	0	0	0	0	0	0	0	0	3.50 \pm 1.6	0	0
4-methyl-3-hexanol	0	0.695 \pm 0.695	0	0	0	0	0	0	0	0	0
4-methyl-3-hexanone	0	5.49 \pm 0.879	0	0	0	0	0	0	0	0	0
1-octen-3-ol	0	0	0.6 \pm 0.6	0	0	0	0	0	0	0	0
3-methyl-2-hexene	0	0	84.1 \pm 9.56	0	0	0	0	0	0	0	0
3-heptanone	0	2.67 \pm 0.32	0	0	0	0	0	0	0	0	0
3,5-dimethyl-2-octanone	14.1 \pm 0.83	0	0	0	0	0	0	0	0	0	0
2-methyl-2-heptenal	6.03 \pm 0.62	0	0	0	0	0	0	0	0	0	0
2-Dodecenal	22.4 \pm 2.12	0	0	0	0	0	0	0	0	0	0
citral variant	0	0	0	3.23 \pm 2.04	0.65	0	0	0	0	0	0
1-octen-3-one	3.34 \pm 0.23	0	0	0	0	0	0	0	0	0	0
3,4-dimethyl-2-hexanone	5.23 \pm 0.60	0	0	0	0	0	0	0	0	0	0
8-methyl-1-undecene	8.53 \pm 0.70	0	0	0	0	0	0	0	0	0	0
3,5-dimethyl-octanone	6.27 \pm 0.34	0	0	0	0	0	0	0	0	0	0
U9.1	0	1.3 \pm 1.10	0	0	0	0	0	0	0	0	0
U9.9	19.2 \pm 0.38	4.81 \pm 1.06	0	0	0	0	0	0	0	0	0
U9.6	0	0	9.3 \pm 7.9	0	0	0	0	0	0	0	0
U10.9	0	0	0	2.67 \pm 2.67	1.28	0	0	0	0	0	0
U14.4	0	0.736 \pm 0.65	0	6.93 \pm 3.76	3.48	0	1.74 \pm 2.59	15.4 \pm 1.3	0	0	2.00 \pm 2.00
Total amount (pg/ul/ant head)	26.2 \pm 0.87	0.24 \pm 0.13	4.08 \pm 0.57	0.37 \pm 0.037	0.20 \pm 0.02	0.44 \pm 0.03	0.024 \pm 0.0053	0.2 \pm 0.07	0.05 \pm 0.01	0.184	0.21 \pm 0.07

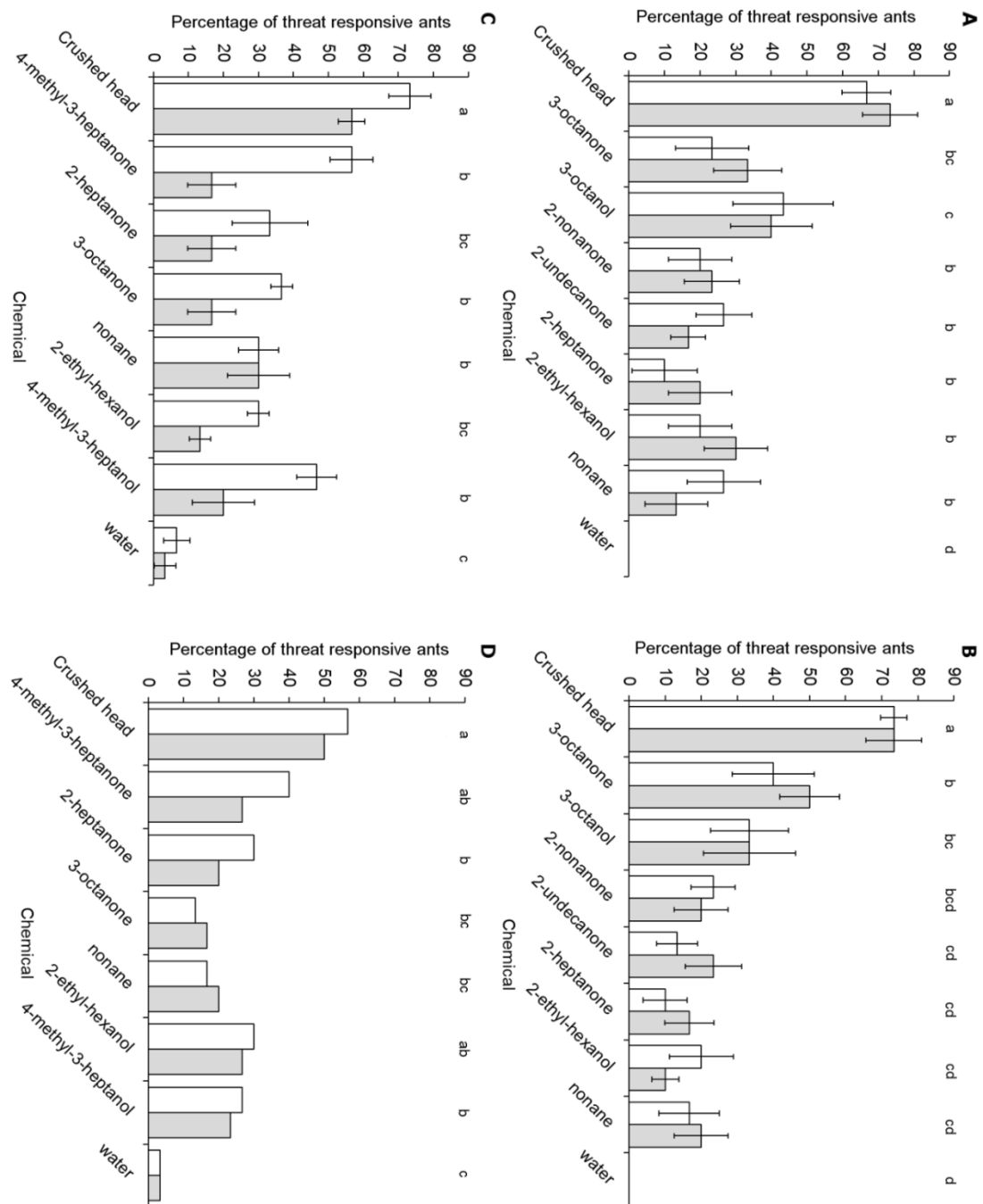


Figure 5.3 Behavioural responses of four species of leaf-cutting ant to the most abundant compounds in their alarm pheromones. Mean \pm s.e percentage of ants showing a positive mandible opening response (MOR) to compounds found in crushed ant heads for 30 small workers (white bars) and 30 large workers (grey bars) sampled evenly between colonies for (A) *Acromyrmex echinator* (5 colonies), (B) *Acromyrmex octospinosus* (5 colonies), (C) *Atta colombica* (5 colonies) and (D) *Atta cephalotes* (1 colony). Different letters above columns indicate treatments that differed significantly from one another at $P < 0.05$ in pairwise comparisons.

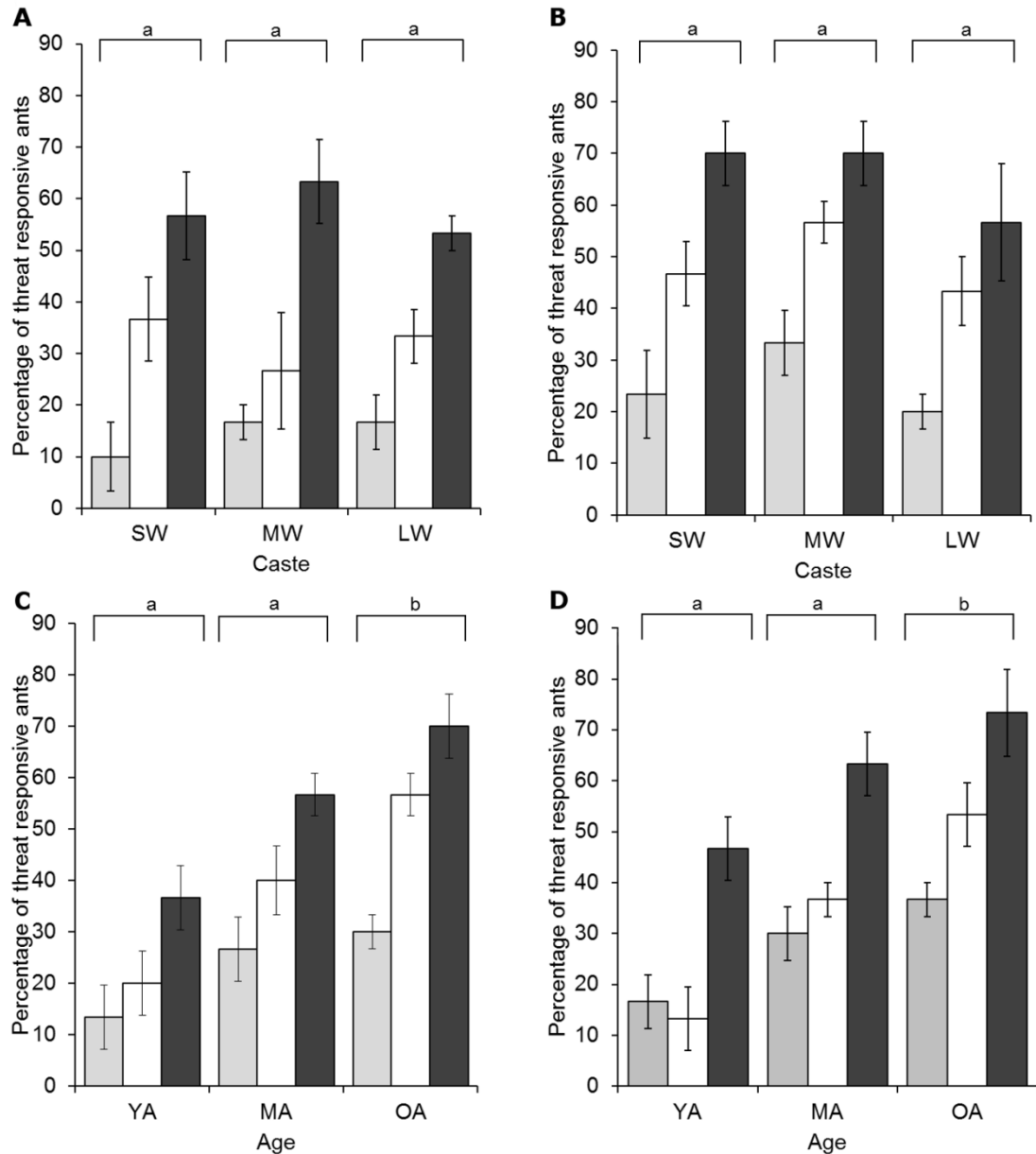


Figure 5.4 The effect of caste and age on the behavioural responses of two *Acromyrmex* leaf-cutting ant species to head-realistic concentrations of the most behaviourally active compounds in their alarm pheromone. Mean \pm s.e percentage of ants showing a positive mandible opening response (MOR)

to solvent negative control (light grey bars), head realistic doses of the most behaviourally active compound (white bars) and crushed nestmate head positive control (dark grey bars). Data are for small workers (SW), medium workers (MW) and large workers (LW) of (A) *Acromyrmex echinator*, and (B) *Acromyrmex octospinosus*, and for young (Y), medium (M) and old worker (O) of (C) *Acromyrmex echinator* and (D) *Acromyrmex octospinosus*. Different letters above sets of columns indicate significant differences between castes or ages of ants at $P < 0.05$ in pairwise comparisons.

5.4.1.4 *Experiment 4: Confirmation of behavioural responses to volatiles released by ants.*

For both *Ac. octospinosus* and *Ac. echinator*, there were significant differences between the treatments in the number of ants showing a MOR ants exhibited ($F_{4,195} = 4.147$, $P = 0.003$, $F_{4,232} = 9.361$, $P < 0.001$ for *Ac. echinator* and *Ac. octospinosus*, respectively). Ants showed the highest frequencies of MOR in response to alarmed nestmates with significantly more gaping their mandibles in response to 1, or 8, nestmates than either a dead nestmate or an empty pot. Identical levels of alarm were produced under red light, simulating darkness for focal ants (Fig. S5.1).

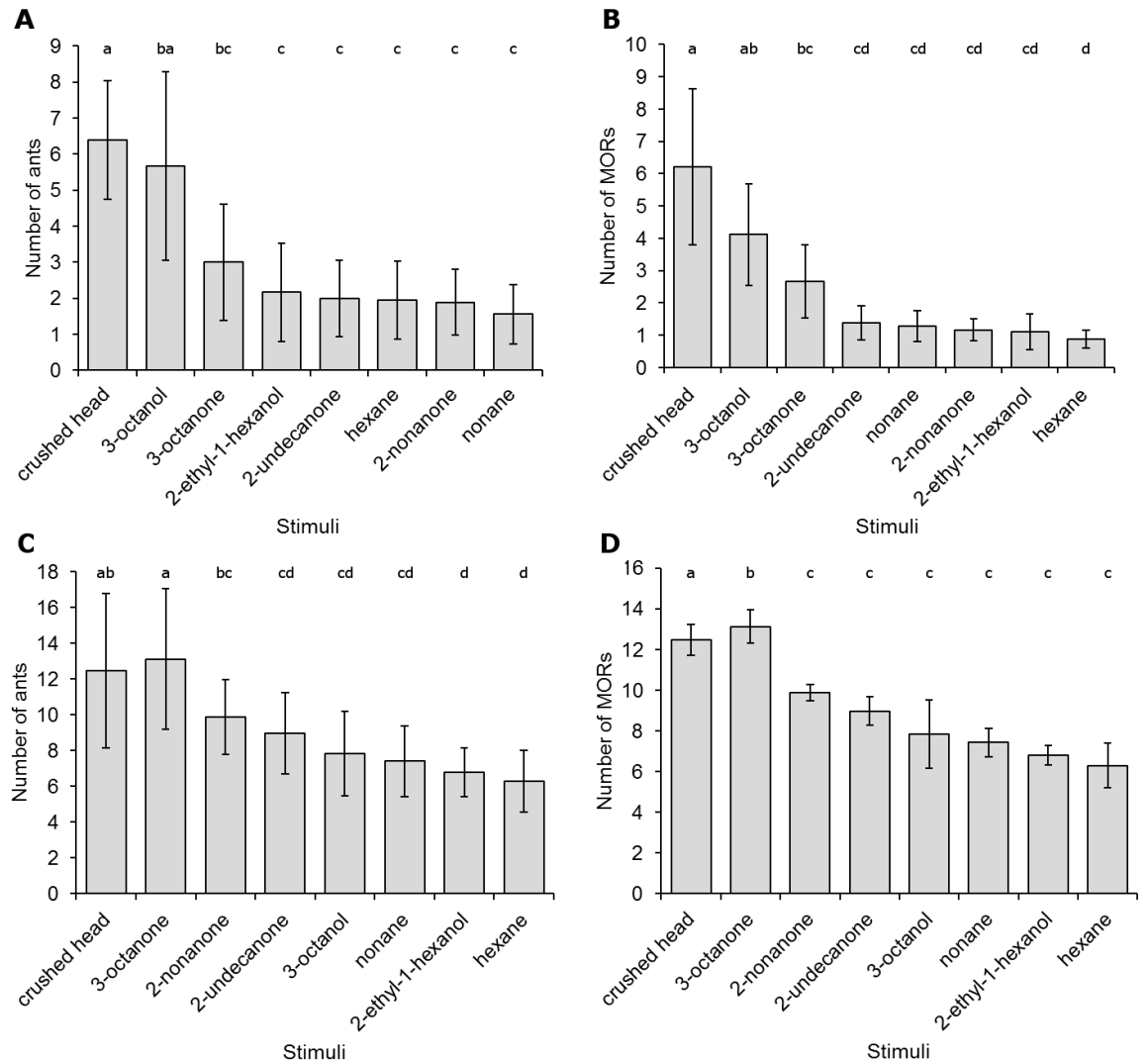


Figure 5.5 Behavioural activity of colonies to compounds isolated from crushed heads. The behavioural responses of both *Acromyrmex octospinosus* (A-B) and *Acromyrmex echinator* colonies (C-D) to the most abundant compounds in their alarm pheromones. Mean \pm s.e. number of ants showing aggregation towards each stimuli (A & C) and number of mandible opening responses observed (B & D). Different letters above columns indicate treatments that differed significantly from one another at $P < 0.05$ in pairwise comparisons.

5.5 Discussion

Contrary to the theoretical prediction of little interspecific variation in alarm pheromone composition, we found composition differed across the attine clade, with 4-methyl-3-heptanone being the most abundant compound in *S. amabilis* as well as *Atta*, but *Acromyrmex* utilising different behaviourally active components. Furthermore in

contrast to previous studies on *Atta*, we found that *Acromyrmex* workers show no caste differences in behavioural response to alarm pheromone, but do exhibit behavioural differences based on age. No individual compound elicited the same level of behavioural response as the natural pheromone, with the most active compound in each species eliciting a response of between 60-90% of that to natural pheromone, suggesting possible additive effects of individual compounds in eliciting full alarm responses.

Although there is a general prediction for a lack of interspecific specificity in alarm pheromones and cues across the animal kingdom (Blum 1969; Hazlett 1994; Vander Meer and Alonso 1998; Commens and Mathis 1999; Laforsch et al. 2006), it might be expected that taxa with larger more complex societies might evolve ‘better’, more informationally rich, mixtures of alarm pheromone (Wilson and Regnier 1971; Hölldobler 1995). This could occur because of the greater quantity of resources they have to defend, and the greater availability and specialisation of workers they have to respond (Hölldobler and Wilson 1990). The dramatic increase in colony size and complexity across the attines, from tens of monomorphic workers in basal taxa to millions of polymorphic workers in *Atta* (Weber 1972; Wilson 1983), would therefore predict changes in alarm pheromone composition. However, the results provided no support for either prediction. The composition of the mandibular gland pheromone differed little in complexity across the attines, and while the main compound in both *Atta* leaf-cutting species was 4-methyl-3-heptanone in keeping with previous studies of *Atta* (Moser et al. 1968; Riley and Silverstein 1974; Hernandez et al. 1999; Hughes et al. 2001a; Francelino et al. 2006), this was also the most abundant compound in *S. amabilis*, while the other leaf-cutting ant genus *Acromyrmex* lacked this compound and appears to use instead 3-octanone or 3-octanol. *T. cornetzi* and *Ap. pilosum* showed entirely unique main constituents to the other attines, and although we found very small

traces of the 4-methyl-3-heptanone, 3-octanol and 3-octanone that were previously found in *T. septentrionalis* and *T. seminole* (Crewe and Blum 1972), the most abundant peaks for these two attines were 3-methyl-2-hexene and 2-dodecenal. One potential explanation for these interspecific differences may be that the components of the mandibular gland secretion serve other purposes, such as being antimicrobial (North et al. 1997). Alternatively they may have evolved to allow some interspecific recognition, as *Acromyrmex* and *Atta* are sympatric competitors, or may be an epiphenomena of differences in diet. The case here is particularly interesting within the genus *Acromyrmex* as compounds are found in the same abundance between these sympatric species but show differing behavioural activity (3-octanone and 3-octanol being most behaviourally active respectively for *Ac. octospinosus* and *Ac. echinator*). Regardless of the reason, it seems that there can be remarkable interspecific variability in the composition of alarm pheromone even between closely related taxa, and further investigation of both the proximate and ultimate explanations for this would be worthwhile.

There was polyethism in the propensity of *Acromyrmex* workers to respond to alarm pheromone. For both *Ac. echinator*, and *Ac. octospinosus*, there was no difference in mandible opening responses (indicating a focal ant has perceived a threat) between morphological castes (small, medium and large age-matched workers), but there were differences between size-matched young, medium and old workers. Differences in alarm behaviour between morphological castes are known in *Atta* leaf-cutting ants, both in the production of alarm pheromone and behavioural responses to it (Hernandez et al. 1999; Hughes et al. 2001a; Francelino et al. 2006). Smaller workers in *Atta* respond at higher frequency to conspecific intruders and are more responsive to alarm pheromone (Wilson 1980; Whitehouse and Jaffe 1996; Hughes and Goulson

2001), something which our behavioural work supports. *Acromyrmex* leaf-cutting ants share many similarities with *Atta*, including worker polymorphism that affects division of labour, so the lack of an effect of size on response to alarm pheromone in *Acromyrmex* is at first sight surprising. Previous MOR studies with *Acromyrmex* have similarly found no difference between physical castes in threat responsiveness (Norman et al. 2014). It may be that the lack of morphological caste differences with respect to nest defence is indicative of *Acromyrmex* having simpler division of labour than *Atta*, or because individuals express a far less extreme alarm response.

The age of workers, even more than morphological caste, is an important and widespread basis for division of labour in social insects, including in *Acromyrmex* leaf-cutting ants (Seeley 1982; Wakano 1998; Camargo et al. 2007; Waddington and Hughes 2010). In both *Acromyrmex* species tested here, age significantly affected the propensity of individual to show an MOR towards chemical stimuli (with older ants responding more frequently). Older *Ac. echinator* individuals are also more threat responsive than young individuals to non-nestmates, and similar relationships with older workers being more aggressive or threat responsive have been reported from many other social insects (Robinson 1987b; Alaux et al. 2009; Waddington and Hughes 2010; van Wilgenburg et al. 2010; Tibbetts et al. 2013; Norman et al. 2014; Santoro et al. 2015). This relationship is likely to be beneficial in insect societies because age polyethism results in older workers spending more time outside of the nest where they are more likely to encounter potentially dangerous threats (Moore et al. 1987; Allan et al. 1987). The proximate physiological mechanism may be that young ants require a period of time to develop the baseline “nestmate” template that they then compare other stimuli to (Gamboa 1978; Sturgis and Gordon 2012a). Alternatively, it may be that younger ants are less threat responsive because their longer potential lifespan makes them more valuable to the

colony than older workers, which will be spending more time outside the nest carrying out behaviours more relevant to alarm cues (Camargo et al. 2007).

The surprising level of interspecific differences seen across the attines in this study highlights the need for future, detailed, comparative work on the alarm pheromones of fungus-growing ants in particular, as well as social insects in general. It will be very interesting to discover whether the logical premise of low interspecific variation in alarm pheromone composition generally holds true, and if not, then why not. The simplicity of alarm pheromones and the interspecific variation composition which they nevertheless show offers much potential as a system for understanding the proximate and ultimate basis for the production of pheromones.

6 The effects of disturbance threat on leaf-cutting ant colonies: a laboratory study



Figure 6.1 Does threat disturbance alter caste ratios or behavioural phenotype of colonies? An aggressive soldier and minor worker of *Atta cephalotes*.

6.1 Abstract

The flexibility of organisms to respond plastically to their environment is fundamental to their fitness and evolutionary success. Social insects provide some of the most impressive examples of plasticity, with individuals exhibiting behavioural and sometimes morphological adaptations for their specific roles in the colony, such as large soldiers for nest defence. However, with the exception of the honey bee model organism, there has been little investigation of the nature and effects of environmental stimuli thought to instigate alternative phenotypes in social insects. Here we investigate the effect of repeated threat disturbance over a prolonged (17 month) period on both behavioural and morphological phenotypes, using phenotypically plastic leaf-cutting ants (*Atta colombica*) as a model system. We found a rapid impact of threat disturbance on the behavioural phenotype of individuals within threat-disturbed colonies becoming more aggressive, threat-responsive and phototactic within as little as two weeks. We found no effect of threat disturbance on morphological phenotypes, potentially because constraints such as resource limitation outweighed the benefit for colonies of producing larger individuals. The results suggest that plasticity in behavioural phenotypes can enable insect societies to respond to threats even when constraints prevent alteration of morphological phenotypes.

6.2 Introduction

The ability of organisms to respond flexibly to their environment is fundamental to their evolutionary success. Adaptation towards a locally optimal phenotype can increase both direct and indirect fitness (Via and Lande 1985; Lande 1985). One way in which this can occur is via the production of size variation in response to environmental

conditions, such as climate or competition, which is seen in a wide variety of organisms found over an environmental gradient (Rosenzweig 1968; McNab 1971; Lomolino 2005). However, many organisms show other morphological and behavioural adaptations to environmental pressures that enable individuals to increase their fitness over the course of their lifetime (Boag and Grant 1981; Engel and Tollrian 2009; Torres-Dowdall et al. 2012). Understanding the biotic or abiotic environmental stimuli involved in regulating the modification of the morphological or behavioural phenotype, and any potential negative implications associated with this, are central to our understanding of the evolution and dynamics of phenotypic plasticity.

Some of the most extreme examples of phenotypic plasticity are provided by the social insects. In these societies it is often thought that the specialization of individuals into behavioural and sometimes morphological phenotypes (castes) may make them better adapted to their particular roles in the colony, thereby enhancing the division of labour that is commonly thought to be key to their evolutionary success (Oster et al. 1978; Bourke and Franks 1995). However, the degree to which specialists actually outperform generalists is still highly contentious and our understanding of these processes remains limited (Dornhaus 2008; Wright et al. 2014; Gordon 2015). Individual workers are more-or-less sterile in species with advanced societies, such as *Atta* leaf-cutting ants, and therefore gain their fitness indirectly, meaning that selection may act simultaneously at both the individual and colony level, such that individual-level and colony-level optimal caste ratios can be hypothesised to be the same (Hamilton 1964; West-Eberhard 1989; Korb and Heinze 2004). The morphological phenotype of adult social insects is determined during development in the eusocial Hymenoptera (ants, some bees and some wasps). Both morphological and indeed behavioural phenotypes are not simply determined by environmental conditions such as

nutrition and temperature (Wheeler 1986; Kamakura 2011), or by genotype (Robinson and Page 1988; Hughes et al. 2003; Smith et al. 2008b; Waddington et al. 2010), but rather by the interaction of genotype and environment (Chapman et al. 2007; Hughes and Boomsma 2007; Schwander et al. 2010). Surprisingly, however, we still have only a limited understanding of how environmental conditions can drive the specialization of different individuals. It is well established that social insect colonies vary in their frequency distributions of morphological phenotypes or behavioural phenotype profiles (Yang et al. 2004; Wray et al. 2011; Chapman et al. 2011; Scharf et al. 2012; Pinter-Wollman et al. 2012; Gordon et al. 2013; Jandt et al. 2014; Wiernasz et al. 2014; Wills et al. 2014). However, our understanding of the environmental conditions generating such intercolony variation is still limited (Hui and Pinter-Wollman 2014; Keiser et al. 2014; Jandt et al. 2014).

Honey bees appear to show differing behavioural phenotypes corresponding to length of disturbance, with short-term disturbance causing an upregulation in aggression (Couvillon et al. 2008), but long-term disturbance decreasing aggressive responses (Rittschof and Robinson 2013a). Environmental factors, such as competition, or indeed predation, are also thought to be important in morphological phenotype production in other eusocial insects such as polyembryonic wasp, termite and social aphid species which show a morphologically specialized soldier caste (Crespi 1992; Shibao 1998; Harvey et al. 2000; Shingleton and Foster 2000; Thorne et al. 2003; Smith et al. 2010). In one of the only direct experimental investigations in ants, Passera et al. (1996) demonstrated that exposure to volatiles from non-nestmate potential competitors caused colonies of *Pheidole pallidula* ants to produce more soldiers within a remarkably short seven week period. However, the environmental stimuli that interact with genotype to

produce different morphological or behavioural phenotypes in other social insects are unknown.

Here we test experimentally the effect of a controlled environmental stimulus of threat-disturbance on morphological and behavioural phenotypes in phenotypically plastic *Atta* leaf-cutting ant societies. *Atta* are one of the most polymorphic of all ant species, with larger workers including specialised soldiers playing the primary role in defending their colonies against threats (Wilson 1980; Whitehouse and Jaffe 1996; Hernández et al. 2002; Hölldobler and Wilson 2010). We test whether repeated threat disturbance leads to colonies adjusting their behavioural phenotype, production of morphological phenotypes, or both. We also explore if other traits are affected by such threat-disturbance.

6.3 Methods

6.3.1 Colony collection

Immature *Atta colombica* colonies, approximately 12 months old, were collected from Gamboa, Panama in May 2013. At this age colonies are too small to produce soldiers (Weber 1972). All colonies were kept in a controlled environment room at the University of Sussex at $80 \pm 5\%$ relative humidity, $26 \pm 2^\circ\text{C}$ and a 12:12 h light/dark cycle, and fed twice weekly on privet leaves (*Ligustrum* spp.) placed in a foraging pot (100 mm x 80 mm x 60 mm), with water provided *ad libitum*. The mutualistic fungus gardens were housed in plastic boxes (115 mm x 75 mm x 75 mm) covered with a flower pot to maintain a dark and humid environment. The 13 colonies were randomly assigned to either a threat disturbance (6 colonies) or control treatment (7 colonies) group. The colonies were of similar size at the start of the experiment (mean \pm s.e.

volume of fungus garden: threat-disturbance colonies 107 ± 23.9 ml and control colonies 156 ± 39 ml) and did not differ significantly in size throughout the experiment (fig. S3). At the start of the experiment no soldiers or large workers (> 2 mm head width) were observed in any of the colonies. Colonies undergoing the threat disturbance treatment had their fungus gardens exposed by removing the flower pot and plastic box lid (85 mm depth) for 2 min. Preliminary trials suggested that this exposure protocol produced a maximal alarm response (increased activity and mandible gaping from workers indicating a response to a threat) from the ants within the 2 min period. The disturbance was carried out on 4-5 days per week for 17 months, while control colonies were not disturbed in this way. Exposure of the vulnerable fungus garden and brood in this way would only occur in nature during a vertebrate predation attempt (such as by armadillos) and, regardless of cause, would represent a serious threat to colony survival, stimulating a dramatic defensive response in leaf-cutting ant colonies in nature (Wilson 1980; Whitehouse and Jaffe 1996; Rao 2000). The long 17 month period gave colonies ample time to alter the production of morphological phenotypes. Furthermore, given the development time in *Atta* is about 2 months (Weber 1972) and that Passera et al (1996) found changes in caste ratios after only 7 weeks, any changes in morphological phenotype should be present after this time. At the end of the disturbance period, the morphological and behavioural phenotypes of colonies were determined. After the end of this long-term experiment we also carried out a shorter disturbance experiment to test at a finer-scale how quickly colonies could upregulate and down-regulate their behavioural responses.

6.3.2 Alteration of morphological phenotype

To determine if threat-disturbance resulted in colonies producing larger individuals, the 50 largest workers from each colony after the 17 month disturbance period were photographed dorsally using a Canon EOS 350d dSLR camera and Canon EF 100 mm f/2.8 Macro lens under constant lighting conditions. Images were imported into Image J and the size of each individual was quantified by measuring the width of the head between the eyes, a commonly used measurement of size in ants including *Atta* (Wilson 1980; Hölldobler and Wilson 1990; Hughes and Goulson 2001; Holman et al. 2011). The colonies were too young and small to produce soldiers in significant numbers, but we also counted any soldiers present to compare the numbers of soldiers between treatment and control colonies.

6.3.3 Alteration of behavioural phenotype

During the last month of the long-term disturbance, assays were carried out to compare behavioural phenotypes of colonies that had either been disturbed, or not, in order to see if threat disturbance affected the responsiveness of ants to threats, we carried out a mandible opening response (MOR) assay (Guerrieri and D'Ettore 2008; Norman et al. 2014). Ants were chilled on ice until immobile and then harnessed in 0.2 ml pipette tips (Starlab, Bucks, UK), cut at the apex through which the ant's head was passed and secured with a thin strip of masking tape. Ants were left for 2 h in the harness to acclimatize before being assayed. Three threat treatments were tested in a random order on each ant: a freshly freeze-killed nestmate, a freshly-killed non-nestmate (*Acromyrmex echinator*) or a burst of carbon dioxide. The latter treatment has been used previously as specific stimulus for sampling defensive workers in *Atta* colonies by simulating a vertebrate predation threat (Wilson 1980; Hölldobler and Wilson 2010). The stimulus ants or carbon dioxide burst were gently placed in contact with an antenna

of the focal ant for 10 s, and the response of the focal ant recorded as either opening its mandibles for > 1s (a positive MOR), or not responding (Norman et al. 2014). For focal ants that showed a positive MOR, the duration of the response was also recorded. For each colony, this assay was carried out for randomly selected soldiers (> 3mm head width), large workers (2-3 mm head width), medium-sized workers (1.2-2.0 mm head width), and small workers (< 1.2 mm head width) to test if specific castes responded differently to defensive stimuli. Five individuals of each caste were tested in each colony, or as many as the colony had for colonies which had very few large workers.

To determine the phototaxis of ants, workers were placed individually in a 90 mm Petri dish, half of which had been covered with black tape (Norman and Hughes 2016). The ants were allowed to acclimatize for 5 min and then filmed for the subsequent 10 min. The proportion of time spent in the light half of the Petri dish was recorded for each individual. This was repeated for 120 ants from threat-disturbed colonies and 140 ants from control colonies (20 per colony in both cases), using randomly selected medium-sized and medium-aged external workers. Worker age correlates positively with a darkening of the cuticular colouration (Armitage and Boomsma 2010), therefore medium-aged ants could be distinguished by their colouration. To see if threat disturbance affected the aggressiveness of ants, individual ants were carefully touched on the head with the tip of a toothpick, similar to Pamminger et al. (2014). The reaction of the ant was ranked (0 = ignore, 1 = antennate, 2 = gape mandibles in a threat response, 3 = bite). This was repeated for 120 ants from the disturbed colonies and 140 ants from the control colonies (20 ants per colony in both cases), using randomly selected medium-sized and medium-aged external workers to control for any differences between castes in aggression. Assays were carried out in the order listed above during the final month of the 17 month disturbance. Ants were

returned to the colony after the assays with at least 5 days occurring between assays.

Given the number of workers per colony (ca. 5000, of which ca. 3,000 would be medias, for colonies of the size used here (Weber 1972)), and that at least 5 days was left between assays, the likelihood of resampling the same ant for multiple assays was very low.

6.3.4 Potential effects on other traits

In order to explore whether changes in the behavioural or morphological phenotypes of individuals in response to disturbance might affect other traits in ways that could be potentially negative, we compared the foraging rate, individual immunity, and brood care propensity of ants from threat-disturbed and control colonies. With the exception of the immunity assays, ants were returned to their colonies after use; given the number of workers per colony and that at least 5 days occurred between assays, it was unlikely that ants were resampled for multiple assays.

Foraging rate was quantified four days following the last feed. The foraging pot of each colony was filled with leaves and the initial mass of the pot recorded. Each pot was then placed back with its respective colony for 1 h, after which the ants within the pots were removed and counted, and the pots reweighed to determine the proportion of leaf material that had been foraged during the 1 h foraging period. This was carried out once for each colony.

To quantify brood care propensity, individual ants were placed in a 90 mm Petri dish with a randomly selected nestmate larva, allowed to acclimatize for 5 min and then filmed for 10 min. The proportion of time spent interacting with larvae during the 10 min period was recorded. This was repeated with 20 medium-sized and medium-aged external workers from each colony. Using external workers avoided the disruption to

the fungus chamber that sampling within-nest workers would have involved, and external workers have been shown previously in many ant species to pick up brood found outside of the nest and transport it back into the colony (Robinson et al. 2012; Tragust et al. 2013).

To determine the effect on individual-level immunity, we measured levels of the phenoloxidase (PO) and prophenoloxidase (PPO) immune enzymes in haemolymph. Haemolymph samples of 1 µl were collected from individual, freeze-killed ants using a calibrated, pulled glass capillary inserted under the cuticle of the thorax. Haemolymph was diluted 1:40 in ice-cold sodium cacodilate/CaCl₂ buffer (0.01 M Na-Cac, 0.005 M CaCl₂), flash frozen in liquid nitrogen and stored at -80°C to disrupt haemocyte membranes and release cellular PPO. All reactions were prepared in 96-well plates on ice. 15 µL of diluted sample was placed in an individual well, together with 5 µL of distilled water for PO reactions or 5 µL of the activation agent alpha-chymotrypsin (5mg mL⁻¹, in distilled water; Sigma Aldrich™) for PPO reactions. Samples were then incubated for 5 min at room temperature. To start the reaction 35 µL of L-DOPA (4 mg mL⁻¹ in distilled water; prepared freshly and protected from light, Sigma Aldrich™) was added to each well and the plate placed in a Molecular Devices VersaMax micro-plate reader. Temperature was set to 30°C and the absorbance of each sample at 492 nm was measured every 15 s over a period of 45 min using SoftMax Pro software. For each sample the enzyme activity was calculated at the maximum slope (V_{max}) in the linear phase of the reaction (usually 200-1000 s after the start of the reaction). Each plate had a control well, which contained only buffer and no sample, and all controls displayed essentially no enzyme activity during the reaction (<0.1 mOD min⁻¹). Two technical replicates were carried out of each reaction and all samples where the reaction curved

showed irregularities were excluded, leaving measurements of PO and PPO for 58 and 67 ants from disturbed colonies, and 50 and 63 ants from control colonies, respectively.

6.3.5 Speed of behavioural plasticity

In order to see how quickly *Atta colombica* colonies altered their behavioural phenotype to disturbance we carried out a finer-scale threat disturbance experiment. Four months after the end of the long-term experiment (the length of at least one generation of adult workers (Weber 1972), the remaining seven control colonies were split into equally sized subcolonies (ca. 500ml fungus per subcolony) that were randomly assigned to either short-term disturbance or control group. Colonies were monogynous so the queen-right sub-colony was randomly assigned between treatments. Disturbed subcolonies were disturbed in the same way as in the long-term experiment but for a period of only 2 weeks, with the behavioural phenotype of colonies being determined using the phototactic (N = 126 per treatment group; 18 ants per subcolony), aggression (N = 140 per treatment group; 20 ants per subcolony) and MOR assays using two stimuli treatment of a nestmate and a non-nestmate (N = 126 and N = 136 for the threat-disturbed and control treatment groups respectively; 18-20 ants per subcolony). Colonies were then left undisturbed for a period of 2 weeks after which the assays were repeated with the same numbers of individuals in order to determine if colonies would then down-regulate behaviours to match their prevailing environmental conditions.

6.3.6 Statistical analyses

The size and behaviours of ants were compared between threat-disturbed and control colonies using generalized linear mixed models (GLMM), which included colony-of-

origin as a random factor. The head widths of ants, length of MOR, phototaxis, brood care and proportion of leaves harvested in the foraging assays were compared using models with gamma distributions and log link function, while the propensities of ants to exhibit a MOR or aggressive response were compared with a binomial distribution and logit link function, and aggression scores using a multinomial distribution and probit link function. The number of ants in each foraging pot in the foraging assay was analysed using a Poisson distribution with a log link function. Levels of PO and PPO were both log transformed to ensure normality and analysed in a GLMM with a Gaussian distribution and identity link function. Colony size, measured as volume of fungus garden, was included as a covariate in all models to control for variation in colony sizes. Best fitting models were selected by comparison of AIC values. Overdispersion was checked for in all cases by calculating a dispersion parameter and none of the models were overdispersed. Nonsignificant interaction terms were removed stepwise in all cases to obtain the minimum adequate models. All statistics were performed in SPSS (v.20 SPSS Inc., Chicago, IL, USA).

6.4 Results

6.4.1 Long-term disturbance

There was no significant difference in the size of the largest 50 workers in the threat-disturbed and control colonies ($F_{1, 648} = 0.665$; $P = 0.415$; Figure 6.2a). The number of soldiers produced was minimal over the 17 month experimental period (4 and 8 from all threat-disturbed and control colonies respectively). There were no differences between treatment and control colonies in the numbers of large workers or soldiers in them over the last six months (Fig. S6.4), and no indication anecdotally of differences before that

either. However, there was an effect of the disturbance treatment on the behaviour of ants in the colonies. Ants from the threat-disturbed colonies spent significantly more time in the lightened half of a Petri-dish compared to ants from control colonies ($F_{1, 258} = 17.09$; $P < 0.001$; Figure 6.2b). In the MOR assay, ants from the threat-disturbed colonies were also significantly more threat responsive compared to those from the control colonies ($F_{1, 719} = 4.15$; $P = 0.042$; Figure 6.2c), and individuals that gaped their mandibles did so for significantly longer ($F_{1, 182} = 6.42$; $P = 0.012$; Figure 6.2d). Overall, all ants from both treatment groups, showed significantly different responses between the three stimuli in both MOR propensity and duration ($F_{2, 719} = 17.1$ $P < 0.001$; Fig. S6.1a; $F_{2, 182} = 16.7$; $P < 0.001$; Figure S6.1b, respectively). Size of the focal ant showed no significant relationship with either propensity or duration of MOR response ($F_{3, 719} = 1.08$, $P = 0.36$, and $F_{3, 182} = 0.99$, $P = 0.4$, respectively). In the aggression assay, ants from the threat-disturbed colonies did not show a difference in propensity to be aggressive compared to ants from the control colonies ($F_{1, 128} = 0.634$; $P = 0.427$; Figure 6.2f), but when they did show an aggressive response, they showed a significantly higher aggression score ($F_{2, 256} = 3.39$; $P = 0.035$; Figure 6.2e). Colony size (volume of fungus) showed no relationships with the size of workers, the propensity or duration of MORs, phototaxis or aggression ($F_{1, 257} = 0.912$, $P = 0.340$; $F_{1, 962} = 0.001$, $P = 0.983$; $F_{1, 321} = 0.758$, $P = 0.385$; $F_{1, 258} = 0.546$, $P = 0.461$; $F_{2, 253} = 0.250$, $P = 0.779$, respectively).

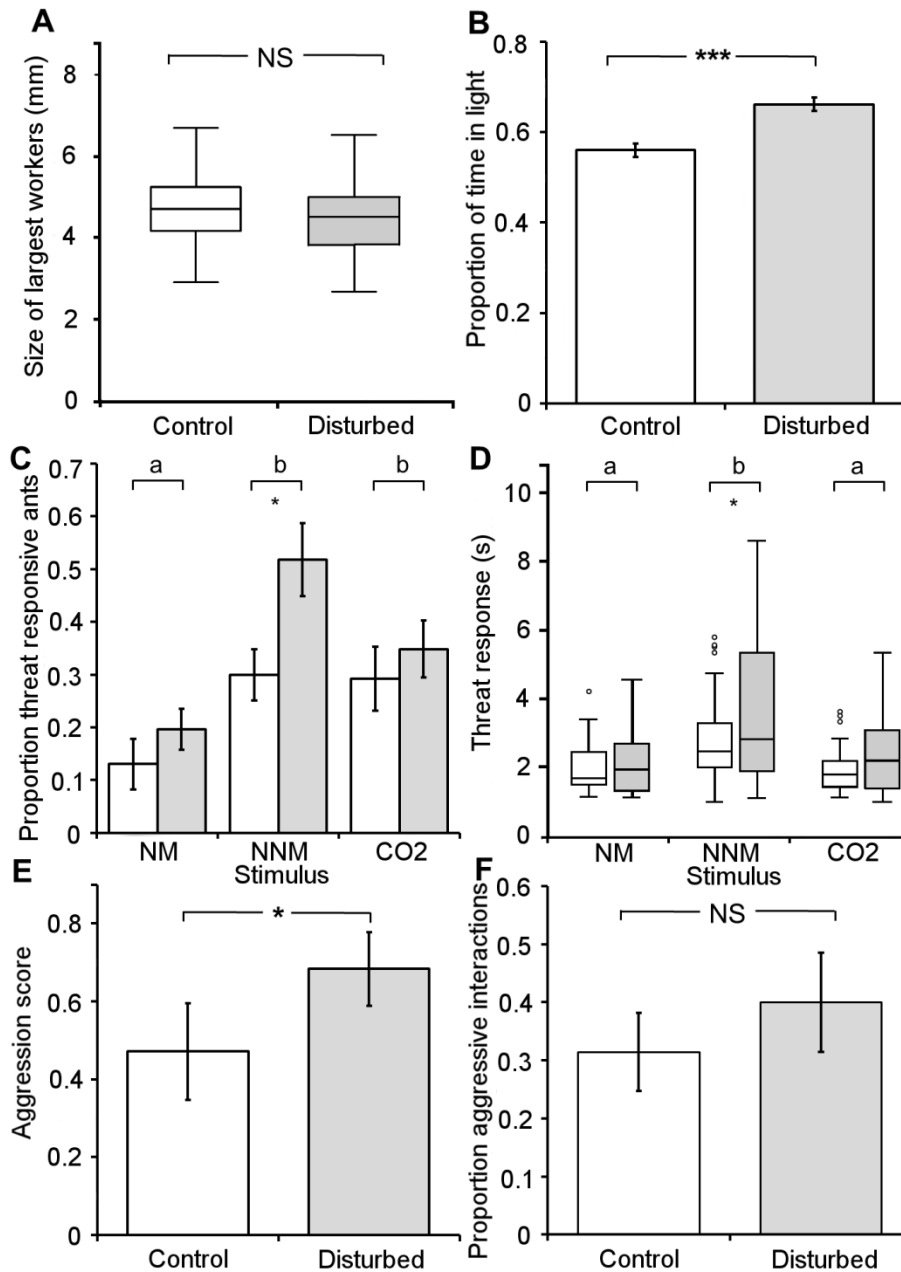


Figure 6.2 Morphological and behavioural phenotypes of long-term threat-disturbed colonies. The mean \pm s.e. morphological and behavioural phenotypes of *Atta* leaf-cutting ant workers from threat-disturbed colonies (grey) and control colonies (white): (A) head width of the 50 largest workers in each colony, (B) proportion of time spent in the light half of a half-blackened Petri-dish, (C) proportion of positive mandible opening responses (MORs) to threat stimuli, (D) length of MOR to three threat stimuli (different letters indicate significantly different responses between treatment stimuli), (E) aggression score (ranging from 0 = no aggression to 3 = bite), and (F) proportion of aggressive interactions. Colonies either received a substantial threat-disturbance every week for 17 months or were not disturbed in this way (control colonies). Asterisks indicate a significant difference between threat-disturbed and control colonies (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$) while different letters above columns in C and D indicate behavioural stimuli which differed significantly from one another in pairwise comparisons.

In the assays exploring potential effects on other traits, there was no difference between the threat-disturbed and control colonies in the number of ants in the foraging pots after 1 h ($F_{1,11} = 0.172$; $P = 0.686$; Figure 6.3a), but the threat-disturbed colonies nevertheless harvested significantly less leaf material than the control colonies during the 1 h period ($F_{1,11} = 31.8$; $P < 0.001$; Figure 6.3b). Colony size showed no significant relationship with either the number of ants in the foraging pot, nor (marginally) on the amount of leaf material they harvested ($F_{1,10} = 2.16$; $P = 0.172$; $F_{1,10} = 4.67$, $P = 0.056$, respectively). There was no significant effect of disturbance on the immunocompetence of ants in terms of either PO or PPO activity ($F_{2,105} = 0.165$; $P = 0.848$, and $F_{2,126} = 0.144$; $P = 0.866$; Figure 6.3c), nor on the propensity of ants to pick up brood ($F_{1,248} = 0.076$; $P = 0.784$; Figure 6.3d). There was no significant difference in size of colonies at the end of the experiment ($F_{1,11} = 0.20$; $P = 0.663$; mean \pm s.e. size of threat-disturbance and control colonies were 1087 ± 182 ml and 1176 ± 120 ml of fungus garden, respectively), or over the course of the experiment (Figure S6.3).

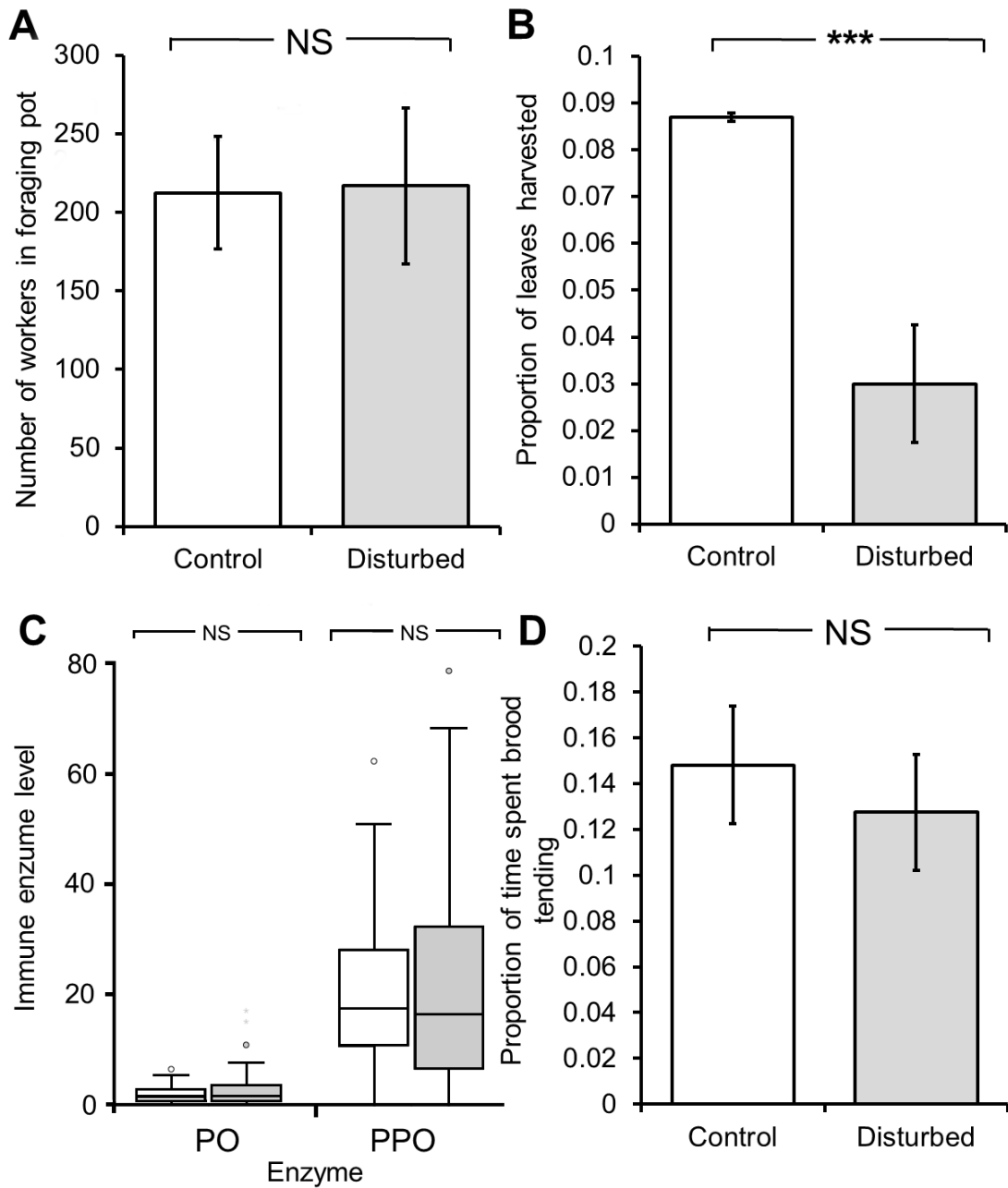
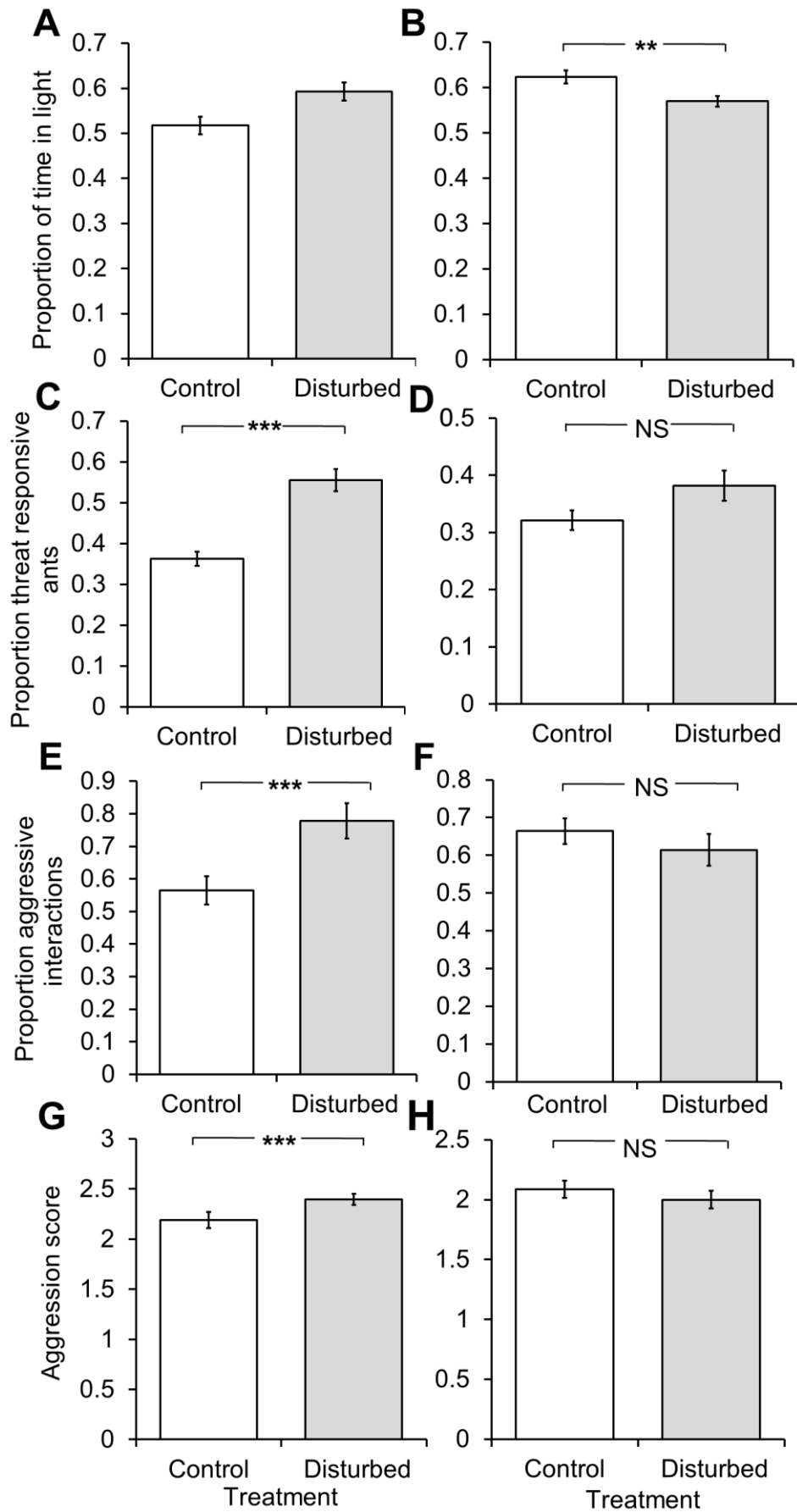


Figure 6.3 Effects of threat disturbance on other traits. The mean \pm s.e. costs of phenotype adaptation to threat disturbance: (A) number of ants present in a foraging area after 1 h, (B) percentage of leaf material harvested after 1 h (foraging efficiency), (C) activity of the phenyloloxidase (PO) and prophenyloloxidase (PPO) immune enzymes, and (D) the percentage of time ants spent showing brood care behaviours over a 10 min period. Colonies either received a substantial threat-disturbance every week for 17 months or were not disturbed in this way (control colonies). Asterisks indicate a significant difference between threat-disturbed and control colonies ($P < 0.001$)

6.4.2 Speed of behavioural plasticity

After two weeks of disturbance, ants from disturbed subcolonies spent significantly more time in the light half of a Petri dish and were significantly more threat responsive than ants from control colonies ($F_{1, 250} = 6.36$, $P = 0.012$, Figure 6.4a and $F_{1, 527} = 20.6$, $P < 0.001$, Figure 6.4c, respectively). They were also significantly more aggressive than ants from control colonies, both in the propensity to show an aggressive response ($F_{1, 278} = 14.15$; $P < 0.001$; Figure 6.4e) and in the aggressiveness of responses ($F_{2, 184} = 3.19$; $P = 0.044$; Figure 6.4g). Two weeks after this short-term disturbance had ended, there was no difference between ants from disturbed and control colonies in their threat response behaviour ($F_{1, 530} = 1.91$; $P = 0.179$; Figure 6.4d), propensity to be aggressive ($F_{1, 278} = 0.758$; $P = 0.385$; Figure 6.4f) or aggression score ($F_{1, 176} = 0.671$; $P = 0.414$; Figure 6.4h), and ants from disturbed colonies spent less, not more, time in the light half of a Petri-dish ($F_{1, 250} = 7.98$; $P = 0.005$; Figure 6.4b).

Figure 6.4 Behavioural phenotypes of short-term threat disturbed colonies (*overleaf*). The mean \pm s.e. behavioural phenotypes of *Atta* leaf-cutting ant workers from threat disturbed colonies (grey bars) and control colonies (white bars) after short term disturbance. Left hand graphs (A, C, E & G) indicate behavioural assays after two weeks of threat disturbance. Right hand graphs indicate (B, D, F & H) indicate behavioural phenotypes two weeks after disturbance had ended. Figures (A) and (B) show proportion of time in the light half of a half-blackened Petri-dish, (C) and (D) the proportion of positive mandible opening responses (MORs) to threat stimuli (pooled responses to nestmates and non-nestmates), and (E) and (F) the average proportion of aggressive interactions and (G) and (H) the average aggression score. Colonies either received a substantial threat-disturbance every day for two weeks or were not disturbed in this way. Asterisks indicate a significant difference between threat-disturbed and control colonies (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).



6.5 Discussion

Repeated threat disturbance of colonies over a prolonged period did not affect the investment by small leaf-cutting ant colonies into morphological phenotypes, but it did alter the behavioural phenotypes of colonies. Ants from disturbed colonies were significantly more threat responsive, aggressive and phototactic than ants from control colonies, and this change in behavioural phenotype occurred after as little as two weeks of threat disturbance, showing how rapidly social insect colonies can behaviourally buffer themselves in the face of environmental perturbation.

The lack of any effect of frequent, and quite substantial, nest disturbances over such a prolonged period of time (17 months) on the production of soldiers or the size of large workers is at first sight surprising. Colonies of many ant species, including *Atta* leaf-cutting ants, show considerable variation within and between populations in the frequency distributions of morphological phenotypes (Davidson 1978; Oster et al. 1978; Wetterer 1995; Yang et al. 2004; Hölldobler and Wilson 2010; Wills et al. 2014), and it seems plausible that such intercolony variation may at least in part be a response to environmental conditions. *Pheidole* ant colonies have been shown experimentally to produce more soldiers within only seven weeks when under greater perceived threat from competitors (Passera et al. 1996). It is unlikely that the 17 month duration of disturbance in the experiment here was insufficient for a shift in caste investment given that the development time in *Atta* is about two months (Weber 1972), and that the disruption of morphological phenotypes can produce a change in allocation after eight weeks in *Acromyrmex* leaf-cutting ants (Hughes and Boomsma 2007). It is also unlikely that the leaf-cutting ant colonies studied here were not genetically capable of producing larger workers or soldiers, given the relatively high intracolony genetic diversity and genotypic variation in size propensity shown by this species (Helmkamp et al. 2008;

Evison and Hughes 2011; Holman et al. 2011). The lack of a response here to threat disturbance in the morphological phenotype distributions of colonies indicates that some other factor or constraint outweighed the stimulus from the disturbance. The exact cause is unknown, but while all colonies were healthy and old enough to produce larger workers, they were relatively small (Weber 1972). The production of larger phenotypes, particularly soldiers, will require the investment of substantially more resources than smaller workers (Oster et al. 1978; Segers et al. 2015). That resource limitation prevented the colonies from increasing their production of larger defensive individuals is therefore one possible explanation for the results.

Although constraints such as resource limitation may therefore limit the ability of social insect colonies to alter their morphological phenotypes, insect societies have other routes to phenotypic plasticity available to them and in this case showed an alteration of their behavioural phenotype. The results from the MOR assay indicate that all castes upregulate their individual-level threat responsiveness in response to colony-threat disturbance rather than this response being limited to certain castes. Furthermore, medium sized workers also showed an upregulation of aggression, phototaxis and threat response behaviour (Chapman et al. 2011; Bengston and Dornhaus 2014). This behavioural flexibility, particularly where aggressive or defensive behaviours are involved, could therefore offer a more adaptable and plastic alternative to a costly investment in a morphological defensive phenotype (West-Eberhard 1989; Tufto 2000; Sih et al. 2004a). The short-term disturbance experiment showed that the behavioural upregulation was dynamic, being upregulated after only two weeks of disturbance, and down-regulated again with two weeks of the disturbance ending. For most variables, the behavioural phenotype of disturbed colonies two weeks after the disturbance had ended was downregulated to levels similar to those of control colonies, although phototaxis

appeared to be downregulated further. This highlights the highly plastic nature of behavioural phenotypes in social insect colonies as well as the exceptional capabilities of colonies to behaviourally buffer themselves in response to environmental disturbance (Robinson 1992; Pamminer et al. 2011; Gordon et al. 2013; Yan et al. 2014).

In contrast to some other studies (Rittschof and Robinson 2013a), the behavioural upregulation showed no evidence of habituation, with the level of upregulation after 17 months being very similar to that after two weeks. Anecdotally there was no evidence of morphological habituation either, with the number of large workers remaining small in both treatment groups over the course of the experiment (Figure S6.4). The lack of habituation may perhaps be due to the severity of the disturbance in the experiment, and of the predator threat that exposure of the fungus garden would indicate in nature (Rao 2000). Interestingly, there was some evidence from our limited assays that the change in behavioural phenotype may affect other traits, with workers from threat-disturbed colonies harvesting less material than those from control colonies during the brief 1 h foraging assay, in spite of the same number of ants having been recruited to the food. There was no difference in the size of fungus gardens of threat-disturbed and control colonies over the course of the experiment, showing that any effect on foraging rate did not have an effect on colony growth in the competitor-free, food-rich environment of the experiment. It would therefore be interesting to see whether threat-disturbed colonies have lower foraging rate over a longer time period and whether this would impact colony growth either when competitors are present or when food is more transiently available.

This study highlights that changes in behavioural phenotype may offer a more rapid and flexible alternative to changes in morphological phenotypes. Social organisms are particularly interesting in this regard because phenotypes can be expressed at both

individual and group levels (Korb and Heinze 2004; Chapman et al. 2011; Dornhaus et al. 2012), and further investigation of the dynamic relationships between phenotypes and environmental cues is likely to be very useful in elucidating the evolution and dynamics of phenotypic plasticity.

7 Cryptic lineages hybridize for worker production in the harvester ant *Messor barbarus*



Figure 7.1 Social hybridogenesis in *Messor barbarus*. Workers in the harvester ant *M. barbarus* are always the product of intra-lineage matings.

7.1 Abstract

The reproductive division of labour between queen and worker castes in social insects is a defining characteristic of eusociality and a classic example of phenotypic plasticity. Whether social insect larvae develop into queens or workers has long been thought to be determined by environmental cues, i.e. larvae are developmentally totipotent. Contrary to this paradigm, several recent studies have revealed that caste is determined by genotype in some ant species, but whether this is restricted to just a few exceptional species is still unclear. Here, we show that the Mediterranean harvester ant *Messor barbarus* possesses an unusual reproductive system, in which the female castes are genetically determined. Using both nuclear and mitochondrial data, we show that Iberian populations have two distinct, cryptic lineages. Workers are always inter-lineage hybrids whereas queens are always produced from pure-lineage matings. The results suggest that genetic caste determination may be more widespread in ants than previously thought, and that further investigation in other species is needed to understand the frequency and evolution of this remarkable reproductive system.

7.2 Introduction

Phenotypic plasticity is a widespread and fundamental trait that enables organisms to adapt their phenotype during development to prevailing environmental conditions (West-Eberhard 1989). One of the classic examples of phenotypic plasticity is the morphological castes exhibited by social insects. The reproductive division of labour between reproductive queens and non-reproductive workers is arguably the defining trait of the major evolutionary transition to eusociality [2], and the caste destiny of a developing individual determines whether it will achieve fitness directly through its

own reproduction or indirectly by enhancing the reproduction of its relatives.

The development of a diploid egg into a reproductive queen or non-reproductive worker in social Hymenoptera (ants, some bees and some wasps) is generally thought to be determined by environmental cues, with larvae being developmentally totipotent with respect to their caste fate. However, a number of cases of genotypic influences on caste determination have now been reported (Schwander et al. 2010), the most extreme of which are a small number of ant species that exhibit social hybridogenesis (Helms Cahan and Vinson 2003; Helms Cahan and Keller 2003; Fournier et al. 2005; Ohkawara et al. 2006; Pearcy et al. 2011; Schwander and Keller 2012). This remarkable reproductive system involves workers being produced sexually from matings between two lineages or even species, and queens being produced exclusively from either within-lineage matings or in some species by parthenogenesis. It is generally detected by microsatellite genotyping of the worker and new queen (gyne) offspring in colonies, with the genotypes of workers indicating that their parents were from two distinct lineages whereas the genotypes of gynes indicate both their parents were from the same lineage (Helms Cahan and Vinson 2003; Helms Cahan and Keller 2003; Fournier et al. 2005; Ohkawara et al. 2006; Pearcy et al. 2011; Leniaud et al. 2012)[4-9].

Social hybridogenesis has fundamental implications for caste determination because hybrid females are constrained to become workers. This leads to a very strong association between genetic material and reproductive potential, and therefore subsequent genetic caste determination. Social hybridogenesis in social insects is consequently thought to be a rare and exceptional phenomenon (Helms Cahan and Vinson 2003; Helms Cahan and Keller 2003; Fournier et al. 2005; Ohkawara et al. 2006; Pearcy et al. 2011; Leniaud et al. 2012). Here we describe a new case of social hybridogenesis, the Mediterranean harvester ant *Messor barbarus*.

7.3 Methods

The harvester ant *Messor barbarus* is a common species in the dry habitat of the Iberian Peninsula. Colonies contain a single queen and several thousand workers. We excavated 27 partial colonies in Sorbas (Spain) and nine additional colonies in Baul, Pozo Alcón, El Mojonar, Alcaraz, Cáceres (Spain) and Aljezur (Portugal). Samples of workers and, when present, gynes (new queens) and males, were collected for subsequent genetic analyses. We also sampled reproductives (queens and males) during nuptial flights in Baul and Alcaraz (Figure 7.2). We used mitochondrial DNA sequence data and microsatellite markers to infer both population and colony structure across our sampling. DNA was extracted from adult ants with Chelex-100.

For the analysis of mtDNA, a portion of COI was amplified for 37 individuals from the seven sampling sites: one individual from each of 28 colonies covering all sites apart from Baul, plus 1 gyne from the Alcaraz nuptial flight and 8 gynes from the Baul nuptial flight. We amplified COI using the universal primers HCO2198 and LCO1490 (Folmer et al. 1994), and an annealing temperature of 50°C. PCR products were sequenced from both ends. Sequences were checked for quality, aligned and trimmed using CodonCode Aligner 4.1. Maximum likelihood trees were constructed with MEGA7 (Kumar et al. 2016). JModelTest 2 (Darriba et al. 2012) suggested GTR + I + G as the best substitution model based on AIC criterion. Branch support values were obtained by 1000 bootstrap pseudo-replicates. A published sequence of *Messor denticornis* was used as an outgroup (GenBank: JQ742637).

Nuclear DNA variation and pedigree were studied using 4 microsatellite markers (*Ms1a*, *Ms2a*, *Ms2c* and *Ms2d*; (Arthofer et al. 2005)(Arthofer et al. 2005). All gynes and males as well as selection of workers were genotyped. For each colony, the

genotypes of the mother queen and her mates were inferred from workers whenever possible. The effective mating frequencies of queens were estimated from worker genotypes using the bias-corrected estimator of effective number of mates (M_{ep}) proposed by (Nielsen et al. 2003). Although this estimator takes into account sample size, we also estimated effective mating frequencies both including all colonies and using only colonies for which at least 8 workers were genotyped. Because gynes and workers were found to be produced by different types of mating (see results), we estimated the effective mating frequency of queens both based on worker offspring (M_{epW}) and based on gyne offspring (M_{epG}) for those colonies for which both were available. To verify the reliability of our four microsatellite markers to detect different patriline, we first calculated the probability that two random fathers shared the same alleles at all loci (Boomsma and Ratnieks 1996). In Sorbas, where we had good estimates of allele size frequencies, the non-detection error due to two males sharing the same alleles was very low for both lineages ($P_{Mbar1}=0.0086$ and $P_{Mbar2}=0.0030$). F -statistics were estimated using SPAGeDi (Hardy and Vekemans 2002). Observed values were compared with the corresponding frequency distributions when random permutations of the data were performed. To determine the population structure, we excluded all worker genotypes and used inferred parents only. Haploid male genotypes were encoded as diploid by doubling their alleles. The number of groups (K) among reproductives was determined using STRUCTURE (Pritchard et al. 2000). The program was run 10 times for each value of $K = 1-10$ under an admixture model with correlated allele frequencies, with 100,000 Markov chain Monte Carlo iterations and a burn-in period of 20,000. The number of group was investigated using the *ad hoc* d-K method (Evanno et al. 2005). For cross-validation of STRUCTURE results, a genetic-distance-based PCoA was performed with GENALEX v6.5. To explore mating patterns, we

augmented the PCoA with connections between parental genotypes found co-occurring in the offspring.

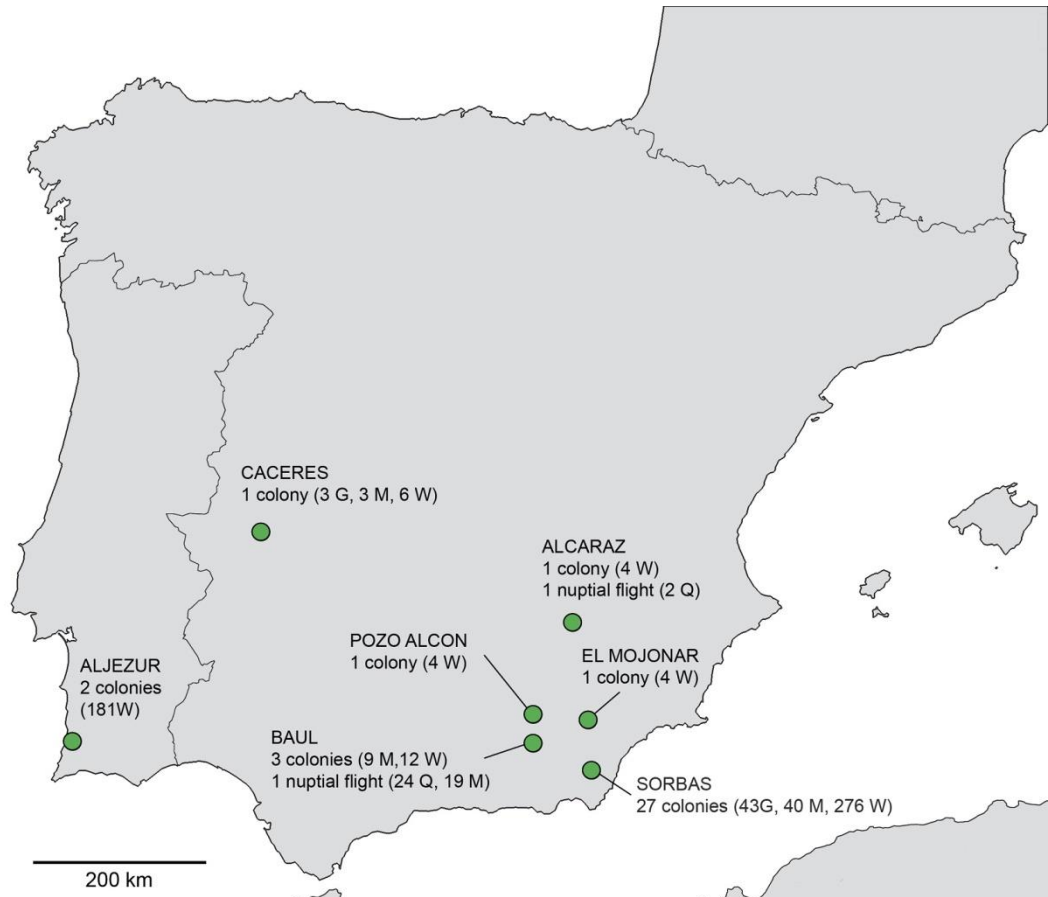


Figure 7.2 Sampling locations of *Messor barbarus* in the South of the Iberian Peninsula (Number of colonies excavated and number of individuals genotyped; Q=mated queens, G=virgin queens, M=males, W=workers).

7.4 Results

Mitochondrial haplotypes clustered into two groups (*Mbar1* and *Mbar2*; Figure 7.3d).

Both were found in Sorbas, Baul and Aljezur, suggesting that the two groups were sympatric across the studied area (Figure 7.2). In the Baul nuptial flight, 20 sexuals were *Mbar1* and 22 were *Mbar2*, while in Sorbas the mother queens of nine colonies were *Mbar1* and of seven colonies *Mbar2*, so in both cases an approximately equal

ratio. We genotyped 634 ants at four microsatellite loci (590 ants from the 36 colonies, plus 44 sexuals from the two nuptial flights; 2.9% missing data). Offspring microsatellite genotypes enabled us to infer the genotype of the mother queen for 30/36 colonies, and paternal genotypes for 29 of these (Table S8.1). The genotype data for each colony were consistent with individuals being the offspring of a single queen that had been inseminated by one or multiple males (see later). There was a significant genetic differentiation between colonies, confirming that all colonies were genuine and that there were no cases of polydomy in the data (global- $F_{ST} = 0.2386$, test by random permutations of individual among pairs of colonies: $P < 0.05$ for all colony pairs). As is usually the case in Hymenoptera, all workers and new queens were found to be produced by sexual reproduction ($N = 491$ and $N = 47$, respectively), whereas males were haploid individuals produced by arrhenotokous parthenogenesis ($N=52$, Figure 7.3)

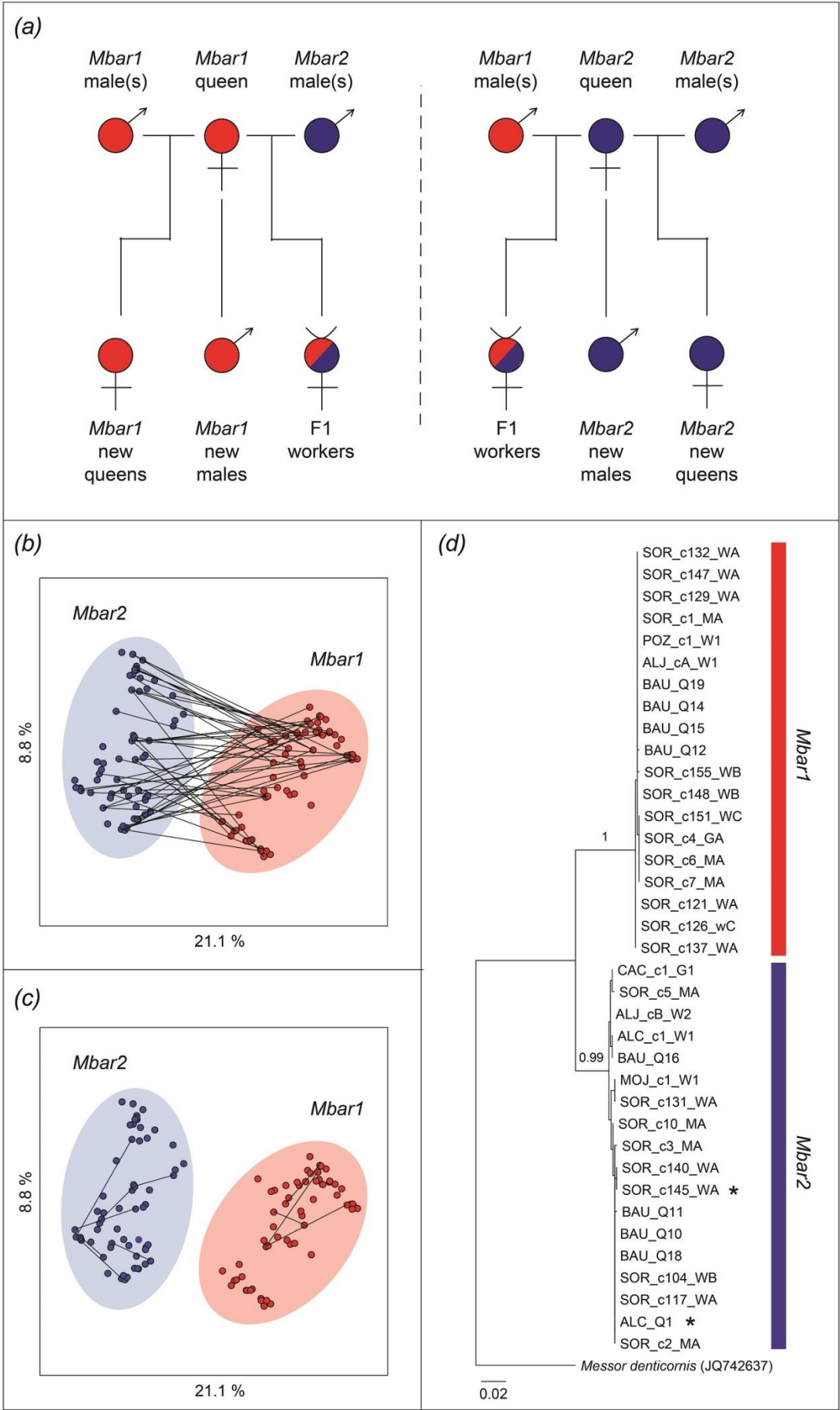
Bayesian clustering analysis using STRUCTURE of reproductive individuals (inferred parental genotypes of colonies, and queens and males from the two nuptial flights; $N = 158$) suggested the presence of two groups. The two groups could be diagnosed based on their genotype at *Ms2c*. The first group consists of reproductive individuals with allele 166 (or rare allele 168) at *Ms2c*, whereas the second group had alleles bigger than 166 only. For 29 individuals, we had ambiguous genotype information at *Ms2c*. Yet, these were consistent with having either allele 166 or alleles bigger than 166 only. We did not find any heterozygous queens with both the allele 166 (or 168) plus a bigger allele. We used the 129 individuals with unambiguous genotype information at *Ms2c* for the following analyses. These two groups were congruent with the mtDNA *Mbar1* and *Mbar2* lineages with the exception of 2/129 individuals (figure 2d). The *Mbar1* and *Mbar2* lineages were highly differentiated ($F_{ST} = 0.28$) at three

microsatellite loci (*Ms2c*, *Ms2a* and *Ms2d*; $P < 0.001$ in each case), and also significantly differentiated at the fourth (*Ms1a*; $P = 0.029$). Each lineage occupied a distinct area of the PCoA plot (Figure 7.3a, c). Remarkably, all gynes (new queens) were found to arise from within-lineage matings ($N = 47$, Figure 7.3c), while all workers were found to arise from inter-lineage matings ($N = 491$; Figure 7.3b). In line with this, the 276 workers genotyped from 22 colonies from Sorbas were more heterozygous than expected under Hardy–Weinberg equilibrium ($F_{IS} = -0.18$, $P < 0.0001$). In all populations, workers were heterozygous at *Ms2c* with allele 166 and a different allele.

The overall mean effective mating frequency of *M. barbarus* based on worker genotypes (M_{epW}) was 2.24 ± 0.16 using all colonies ($N = 27$ colonies), or 2.36 ± 0.22 using only colonies for which at least 8 workers were genotyped ($N = 18$ colonies). The effective mating frequencies based on worker genotypes of *Mbar1* queens were slightly smaller than for *Mbar2* queens (all colonies: *Mbar1* $M_{epW} = 1.91 \pm 0.2$, $N = 15$ colonies and *Mbar2* $M_{epW} = 2.71 \pm 0.22$, $N = 11$ colonies, $t = 2.62$, $df = 24$, $P = 0.015$; colonies for which at least 8 workers genotyped: *Mbar1* $M_{epW} = 2.03 \pm 0.25$, $N = 11$ colonies and *Mbar2* $M_{epW} = 3.01 \pm 0.36$, $N = 6$ colonies, $t = 2.3$, $df = 15$, $P = 0.036$). Intriguingly, the effective mating frequencies of mother queens estimated from their gyne offspring (M_{epG}) appeared significantly smaller than when estimated from their worker offspring (based on colonies for which we had both M_{epW} and M_{epG} ; overall: $M_{epG} = 1.3$ vs. $M_{epW} = 2.3$, $N = 6$ colonies of the two lineages, $t = 2.61$, $df = 5$, $P = 0.048$; for *Mbar2* lineage only: $M_{epG} = 1.36$ vs. $M_{epW} = 2.56$, $N = 5$ colonies, $t = 2.99$, $df = 4$, $P = 0.04$). However, as this is based on only six colonies, five of which were *Mbar2*, and for five of which the numbers of workers or gynes genotyped were small (< 8), it will need further research to determine if this is a genuine difference.

Assuming an equal frequency of *Mbar1* and *Mbar2* in a population (as was found at Sorbas and Baul) and a total effective queen mating frequency ($M_{epW} + M_{epG}$) of $2.24 + 1.36 = 3.6$, the likelihood of a queen of one lineage failing to mate with at least one male of the other lineage (and therefore being unable to produce the workers necessary for a viable colony) can be estimated as $0.5^{3.6} = 8.2\%$. The probability of a queen failing to mate with at least one male of her own lineage (and therefore being unable to produce gyne offspring) is similarly 8.2%. The combined probability of a queen failing to obtain the necessary matings to both produce the workers necessary for a viable colony and produce gynes is then 16.5%.

Figure 7.3 Social hybridogenesis in *M. barbarus* where two genetic lineages (*Mbar1* and *Mbar2*) co-exist within each population (overleaf). (a) Pure lineage matings produce queens whereas inter-lineage matings always produce sterile workers. (b, c) PCoA plot based on genotypes of 129 reproductive individuals (nuclear DNA) augmented with connections between parental genotypes found co-occurring in (b) workers and (c) gynes (new queens). The percentage of variation explained by each PCoA axis is indicated. (d) Maximum-likelihood tree inferred from a portion of COI gene (mitochondrial DNA). Numbers at nodes indicate bootstrap values. Specimen name gives population (SOR: Sorbas, BAU: Baul, POZ: Pozo Alcón, MOJ: El Mojonar, ALC: Alcaraz, CAC: Cáceres, ALJ: Aljezur), colony number and caste. The two individuals with a star symbol are cyto-nuclear mismatches.



7.5 Discussion

Our genetic analyses based on mitochondrial DNA and nuclear DNA revealed the existence of an unusual population structure in the harvester ant *M. barbarus*. Two cryptic lineages (*Mbar1* or *Mbar2*) coexist in Iberian populations. These lineages appear to be inter-dependent because all workers were found to be produced from inter-lineage mating. Despite constant hybridization for hybrid worker production, the lineages are consistently genetically divergent across generations: haploid males are produced asexually and diploid queens are produced by same-lineage mating (figure 2a). Two queens had cyto-nuclear mismatches (mtDNA from one lineage and nDNA from the other one) suggesting that rare introgression events may exist (see (Darras and Aron 2015) for potential mechanisms). Applying molecular clocks of 2.3 to 4% per million years (standard clock and upper bound estimate for insect COI; (Papadopoulou et al. 2010)), the divergence of *Mbar1* and *Mbar2* mtDNA was estimated to be 1.4-2.5 million years. The system itself may, however, be much more recent as the two lineages could have arisen from secondary contact between previously geographically isolated populations or from two different species.

In most ant species, the development of a diploid egg into a reproductive queen or a non-reproductive worker is thought to be determined by environmental cues, although genotypic influences on caste determination have been reported (Schwander et al. 2010). In *M. barbarus*, the caste-genotype association suggests the existence of strong genotypic effects. Such reduced totipotency of hybrid (worker) and non-hybrid (sexual) brood requires queens to mate multiply with males of both lineages to produce a viable colony and achieve reproductive fitness (Anderson 2008). Remarkably, however, observed mating frequencies in *M. barbarus* were found to be low enough that

a large proportion of queens (ca. 16.5%; see electronic supplementary material) likely fail to mate with both males of their own lineage and those of the other lineage if mating is random, and therefore are either unable to produce the workers necessary for a viable colony or are unable to produce gyne offspring. Inter-lineage mating pairs were more numerous than same-lineage pairs (see electronic supplementary material), suggesting that there may be some pre-zygotic sexual selection, something that is relatively unknown in social insects. Nevertheless, there seems likely to be a significant fitness cost associated with the system, implying that there must also be a significant fitness advantage associated with social hybridogenesis for the phenomenon to have evolved. Hybrid vigour of workers is one possibility (Leniaud et al. 2012).

The reproductive system of *M. barbarus* is strikingly similar to that of *Pogonomyrmex* harvester ants, in which inter-dependent lineage pairs also occur (Schwander et al. 2007). These lineages have been shown to harbour chimaeric genomes of two parental species, but the causal link between this hybrid origin and the actual system remains controversial (Sirvio et al. 2011). Lineages of *M. barbarus* may also be of interspecific hybrid origin as unusual colony mixing and hybridization has been previously reported in the genus (Steiner et al. 2011).

Remarkably, social hybridogenesis with a variety of mechanisms has now been reported in seven phylogenetically widespread ant genera: five myrmicines (*Messor*, *Pogonomyrmex*, *Solenopsis*, *Vollenhovia*, and *Wasmania*), and two formicines (*Cataglyphis* and *Paratrechina*) (Schwander and Keller 2012). The multiple independent origins of these systems suggest a general predisposition for the evolution of hybrid workers in ants, but the exact mechanism(s) have remained difficult to untangle. It is notable that three of the cases of social hybridogenesis involve species in arid environments (*Messor*, *Pogonomyrmex* and *Cataglyphis*), and two involve invasive

ants (*Wasmania* and *Paratrechina*; *Volenhovia emeryi* is also an invasive species, though not, it is thought, in the area in which genetic caste determination has been described), suggesting that life-history characteristics may select for the evolution of this remarkable reproductive strategy. Hopefully, the accumulation of genomic data for more ant species will provide some clues to how these unorthodox reproductive systems originate.

8 Behavioural development, fat reserves and their association with productivity in *Lasius flavus* founding queens



Figure 8.1 *Lasius flavus* queens show alternative pathways to reproductive productivity. An attentive *L.flavus* queen shows appropriate brood care behaviours towards her larval offspring.

8.1 Abstract

Reproduction-related behaviours are key components determining individual fitness. Many behavioural traits are linked and such trait associations often affect fitness. Here we combine behavioural and physiological data during two critical time points of founding queens (early and late nest-founding stage) in the claustral ant *Lasius flavus* to assess how these factors affect their initial productivity. We show that most behavioural traits, except brood care behaviour, are plastic during queen development and demonstrate that there are alternative behavioural pathways to achieve high productivity under standardized conditions. These results indicate that queens can utilise multiple behavioural trait combinations to maximise reproductive output at the earliest, and arguably most critical, time of colony foundation.

8.2 Introduction

The way organisms interact with their biotic and abiotic environment is crucial for their reproductive success. This is particularly obvious for behaviours directly associated with reproduction when suboptimal behaviour will have direct, and potentially detrimental, consequences for the fitness of the individual. In species with extended parental care, there will be strong selection for the behavioural responses of the providing individual to be both timely and accurate.

However, optimal parental behaviour might conflict with the behavioural requirements during other parts of the reproductive cycle and hence it may be adaptive to exhibit flexibility in certain behaviours. For example while display, courtship and aggressive behaviours might be beneficial pre-copulation, they could have detrimental

effects when expressed during times of brood care (Wilson and Boelkins 1970; Ritters et al. 2000; Viñuela et al. 2010). As a result, organisms often exhibit sequences of behaviours during which the expression of the appropriate behaviour is restricted to the required time point (Dawson 2007; Golabek et al. 2012).

In recent years it has become apparent that many different behavioural traits can be associated with one another (Sih et al. 2004a; Jandt et al. 2014). While such stable trait associations can be beneficial they might inhibit the maximization of behaviour-related fitness components in different contexts (Sih et al. 2003; Wray et al. 2011; Pinter-Wollman et al. 2012). Studying behavioural sequences and their fitness consequences, including at stages where optimal behaviours for different purposes may be different, is necessary to understand the maintenance of behavioural diversity including contexts where behavioural phenotypes may be sub-optimal (Jandt et al. 2014).

Ant queens offer an excellent model system to investigate such questions in many species because they go through a series of major environmental, and behavioural, transitions in the early stages of their lives from the safe environment of their natal nest to the risky nuptial flight followed by the excavation of their underground nest (Hölldobler and Wilson 1990; Bourke and Franks 1995; Julian and Gronenberg 2002). For claustral species such as *Lasius flavus*, these changes are abrupt because queens never leave their nest after founding it. Therefore, the fat reserves of the queens are the sole source of nutrition for the first set of worker brood. Here, we investigate if the combination of physiology and behaviour changes during the critical nest-founding period of the claustral ant species *Lasius flavus*. We do this at two time points. The first observation point was shortly after the nuptial flight in the early brood tending period. The second observation point was late into the brood tending period when the brood had

almost exclusively pupated and so required less intensive brood care behaviour. We expected that if behaviour is optimized at both time points then queens would show low activity and negative phototaxis (because leaving the incipient nest likely carries high costs in terms of predation risk). Secondly, we expected that brood care and high fat reserves should be strongly associated with productivity, as seen in other ant species.

8.3 Methods

8.3.1 Study organism

Lasius flavus ant queens (n = 28) were collected during their mating flight between 16:00-18:00 on August 31st 2014 in Preston Park, Brighton UK (50°50'30.0"N 0°08'53.8"W) and were immediately weighed on a Precisa 125A balance to the closest 0.0001g then placed in small plastic pots (100 mm x 80 mm x 60 mm), with moist cotton wool at the bottom. Ants were kept in an incubator at $23 \pm 1^\circ\text{C}$ and 100% relative humidity in 24 h darkness to mimic their natural claustral environment (Sommer and Hölldobler 1995). Ant queens had access to water *ad libitum*. All queens had already shed their wings indicating successful mating.

8.3.2 Behavioural traits

Three behavioural assays (activity level, phototaxis and brood care) were carried out to assess the behaviours of queens at two time points (once 4 weeks after collection when queens have both eggs and larvae and then a further 4 weeks later when almost all brood had pupated. These assays were chosen as they correspond to three main axes which can help characterise the behavioural repertoire of individuals in ant societies (Pamminger et al. 2014).

To test the activity level of queens, each individual was placed in a 90 mm Petri dish lined with filter paper, allowed to acclimatise for 2 min and filmed for 5 min using a Logitech c920 webcam. Speed of movement was quantified using Antrak path analysis software (Tranter et al. 2014). To test the phototaxis of queens, each individual was placed in a 90 mm Petri dish lined with filter paper half blackened out with tape. After 2 min acclimatisation, individuals were filmed for 5 min and the proportion of time spent in the light half of the Petri dish recorded. To test the brood care behaviour of queens, individual nest pots were observed for 5 min periods and the amount of time spent by queens on their brood recorded. For all behavioural assays, queens were removed from the incubator and moved to the laboratory. All assays were carried out at the same time of day, at a constant temperature of 23°C.

8.3.3 Productivity measurement

On November 5th 2014, before the queens first winter hibernation, we counted all worker larvae and pupae present in the nest (no sexuals are produced at this stage).

8.3.4 Statistical analysis

To observe if queens were consistent in their behaviours between time point 1 and time point 2 we carried out either a Pearson's or Spearman's test for association for behaviour (depending on whether data were normally distributed) specifying that samples were paired (i.e. non-independent). To investigate potential trait associations at both measured time points we used two principal component analyses (PCA) on the z-transformed response variables (one for each time point). This approach is useful when behavioural traits co-vary and helps simplify data to fewer variables. All principal components (PC's) with an eigenvalue greater than one (explaining more than 70% of

the variation) were used for further analysis (Gotelli and Ellison 2004). These PC's were then modelled against productivity using a GLM with number of brood items as a response variable (following a Poisson distribution). Non-significant interaction terms were removed in a stepwise manner to give a minimum adequate model. The model fit and potential over-dispersion was checked visually using q-q plots and the ratio of residual variation and degrees of freedom. All analyses were performed using R (R.3.2.3, The R Development Core Team, 2014), except the PCA analysis, which was performed in PAST v6.

8.4 Results

8.4.1 Behavioural changes

Out of all three behavioural measures, only brood care tendency was stable over the two time points ($r_s = 0.40$, $P = 0.031$; Figure 8.2), queens were not consistent for either phototaxis ($r_s = 0.23$, $P = 0.23$), or activity level ($T_{27} = 0.87$, $P = 0.39$; Figure S8.1). For correlations between variables within time points see Figure S8.2 and S8.3.

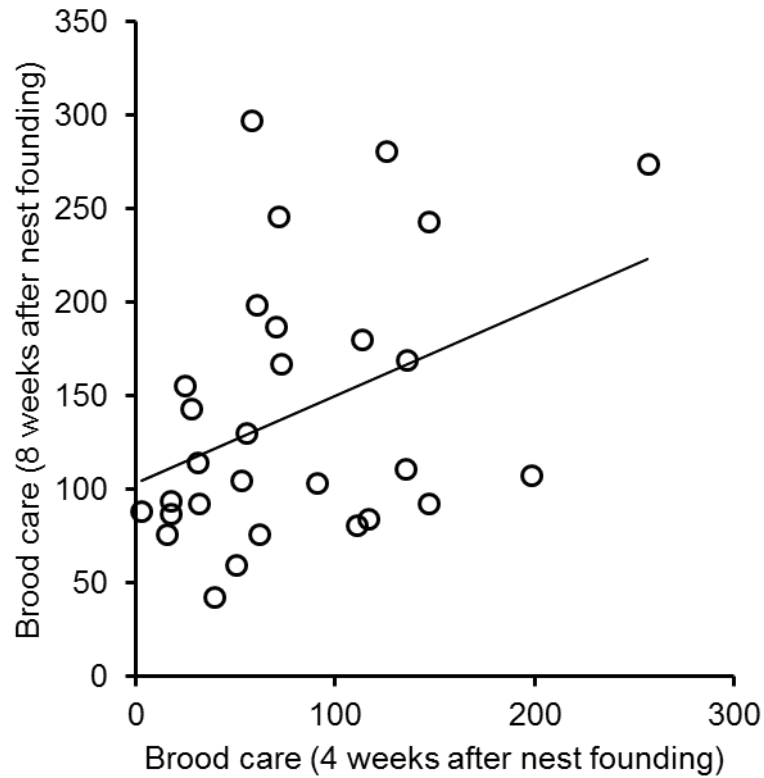


Figure 8.2 Behavioural consistency. Significant behavioural consistency over two time points 4 and 8 weeks post-nest founding for 28 mated *Lasius flavus* queens.

8.4.2 Reproductive productivity

The first PCA for the behaviour of queens at the start of nest founding resulted in two PC's with an eigenvalue greater than one (for those eigenvalues less than one see Table S8.1). The first PC showed a strong positive association between activity and phototaxis (from now on referred to as the activity-phototaxis PC) while the second PC showed an equally strong positive association between initial queen weight and brood tending (from now on referred to as the weight-brood PC; see Table 8.1). Both PC's and their interaction had a marginally significant effect on the productivity of *L. flavus* queens (activity-phototaxis PC: $z = 1.979$, $P = 0.047$; weight-brood PC $z = 2.864$, $P = 0.004$; activity-phototaxis PC x weight-brood PC $z = -1.991$, $P = 0.046$; Figure 8.3).

The second PCA for queen behaviour pre-hibernation yielded three PC's with an eigenvalue greater than 1. The first PC indicated a strong positive association between activity and weight (referred to hereafter as the active-weight PC), the second PC was composed of a strong positive association between brood care and phototaxis (from now on the brood-phototaxis PC) and the third PC showed a negative association between these traits (from now on the brood-light-aversion PC). During model selection all PC's and interactions were shown to have no significant effect on productivity (all $z < 1.69$ $P > 0.091$) except the brood-phototaxis PC which had a significant positive effect on productivity ($z = 2.56$, adj. $P = 0.018$; see Figure 8.3c).

8.5 Discussion

Here, we show that the activity and phototaxis of *Lasius flavus* ant queens exhibits plasticity over two behaviourally relevant time points during the brood care period, while their brood care effort remains consistent. We further demonstrate that different combinations of these behaviours and queen fat reserves predict pre-hibernation productivity. These results suggest that there may be multiple behavioural trait associations that queens can utilise to maximise their fitness. In the early stages of brood care, queens with a combination of high body weight and a strong brood care tendency or, alternatively, high activity and positive phototaxis, achieved higher productivity. The positive association between the former two traits and amount of brood produced is relatively straightforward to explain given that brood care behaviour and high fat content in *Lasius* queens are associated with high reproductive output (Keller and Passera 1989; Fjerdingstad and Keller 2004).

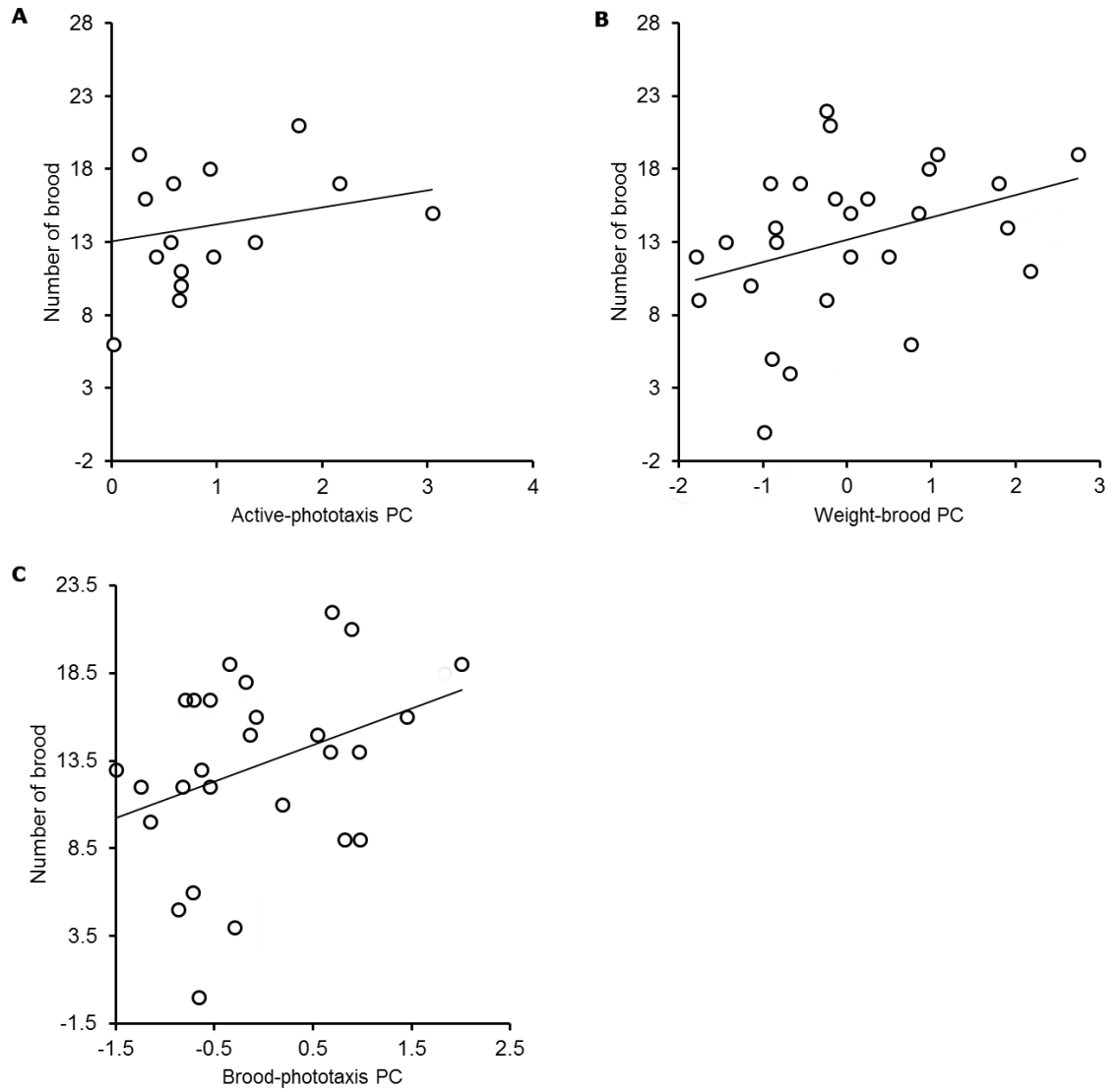


Figure 8.3 Behaviour and productivity. Behavioural characteristics that correlate significantly with initial productivity for 28 mated *Lasius flavus* queens. Response variables represent PCA axes (combinations of the three behavioural traits: brood care [time out of 120 s ant queen spent exhibiting brood care tendency], phototaxis [time out of 120 s spent in a light environment compared to a dark environment] and activity [outputted from AntTrak tracking software], as well as queen weight) that correlate positively with productivity (total brood produced before hibernation) 4 weeks after nest founding (A & B) and 8 weeks after nest founding (C).

Table 8.1: Statistical results from a PCA analysis assessing combinations of three behavioural traits and queen weight at two time periods in colony foundation (4 and 8 weeks post nest founding) for 28 mated *Lasius flavus* queens. We present eigenvalues, percent variation explained and factor loading for all PC's with an eigenvalue bigger than 1 and factor loadings bigger than 0.5 were used in further analysis.

PCA during the first period (4 weeks)	Factor loadings		PCA during the second period (8 weeks)	Factor loadings		
	PC1	PC2		PC1	PC2	PC3
	activity- phototaxis PC	weight- brood PC			brood- phototaxis PC	
Eigenvalue	1.52	1.357	Eigenvalue	1.383	1.032	1.011
% variation	37.995	33.926	% variation	34.574	25.793	25.281
broodcare	-0.178	0.669	broodcare	-0.007	0.7	0.679
activity	0.671	-0.269	activity	0.667	-0.348	0.158
phototaxis	0.685	0.227	phototaxis	0.21	0.604	-0.714
weight	0.22	0.655	weight	0.715	0.153	0.071

Perhaps less intuitive is the positive relationship between activity, phototaxis and productivity. While high activity might be beneficial during nest excavation, positive phototaxis would seem unlikely to be beneficial at this stage and might represent a case of a maladaptive behavioural trait combination (Sih et al. 2003; Wray et al. 2011; Pinter-Wollman et al. 2012). While high activity and positive phototaxis is likely beneficial during the short queen mating flight, these traits are unlikely fitness relevant beyond this time point. The negative interaction between these two trait associations

and the amount of brood produced indicates that these are alternative and mutually exclusive pathways to high reproductive output.

At time point two, when most brood had pupated, a positive association between brood care tendency and phototaxis were the only combination of behaviours to correlate significantly with productivity. While brood care tendency is clearly an important and stable trait during this period, the claustral nature of queens will likely make it impossible for them to express positive phototaxis under natural conditions. Positive phototaxis is known to be vitally important for the nuptial flights, but should be reversed during the post-flight, claustral founding period. The consistent, positive association between phototaxis and productivity could either indicate that positive phototaxis has a non-intuitive (indirect) effect on fitness or alternatively might simply be not strongly selected against since queens will rarely be exposed to light.

In summary, combinations of different suits of behaviours are relevant for pre-hibernation productivity in the lifecycle of *Lasius flavus* ant queens, with brood care tendency being the only stable behavioural trait consistently expressed and associated with high reproductive output. This variation indicates that queens can utilise alternative behavioural strategies to increase reproductive output at the earliest stages of colony foundation.

9 Simple societies and personalities:
behavioural consistency, syndromes
and association with rank in the
primitively eusocial ant *Dinoponera*
quadriceps



Figure 9.1 Do primitively eusocial ants show personalities? An inquisitive, individually marked *Dinoponera quadriceps* poses for a photo.

9.1 Abstract

There is growing recognition of the ecological and evolutionary importance of animal ‘personalities’ or behavioural syndromes, particularly within social insect biology. Such behavioural differences between individuals could be one mechanism for the division of labour between individuals within a colony that is often considered a key determinant of the ecological and evolutionary success of social insects. Social species with simple societies offer an important window into the evolution of personalities, but to date very little work has looked at the relationship between social structure and personality in such societies. We explored personalities in the dinosaur ant *Dinoponera quadriceps*, a species which has secondarily lost the queen caste and reverted from a highly eusocial ancestor to simple societies with a dominance hierarchy headed by a single, mated worker. We investigated if dinosaur ants exhibit personalities or behavioural syndromes, and if these are predictive of rank in the hierarchy. We find that these ants show consistent individual differences in behaviour, i.e. personalities, but find no evidence of behavioural syndromes. We also find that individual personality correlates with rank in the hierarchy, even within the set of relatively high-ranking individuals we examined here, suggesting that the behavioural profile of an individual may influence its position in the social hierarchy. Future, comparative work on personalities in taxa with simple societies will be important for understanding the evolution and maintenance of these phenomena in social animals.

9.2 Introduction

In recent years, numerous studies have found that animals from a wide variety of taxa exhibit individual differences in behaviour that are consistent across time or context

(termed ‘personalities’), or suites of behavioural traits that are correlated across individuals (termed ‘behavioural syndromes; Dingemanse, 2002; Gosling and John, 1999; Gosling, 2001; Jandt et al., 2014; Réale et al., 2007; Sih et al., 2004). Personalities and behavioural syndromes have adaptive value (Dingemanse et al. 2004; Boon et al. 2007; Smith and Blumstein 2007), and may influence fundamental evolutionary processes such as speciation (Wolf and Weissing 2012; Ingley and Johnson 2014).

Social insects are particularly interesting study systems for the investigation of behavioural syndromes and personalities. Research has demonstrated that personality potentially plays an important role in inducing and maintaining the division of labour within social insect societies that is a key feature in their ecological and evolutionary success (Robinson 1992; Beshers and Fewell 2001; Modlmeier and Foitzik 2011; Jandt et al. 2014). Many studies have shown there can be behavioural syndromes or personalities within eusocial insects, both at the individual and colony levels (Réale et al. 2007; Modlmeier and Foitzik 2011; Chapman et al. 2011; Scharf et al. 2012; Pamminger et al. 2014). However, almost all of these studies have focussed on highly eusocial species with complex societies. Very little work has been carried out on species with simple societies, although these may offer an important insight into the evolution of personalities and their relationship with social complexity and subsequent division of labour (Cant et al. 2006).

There is some evidence from other social species that group or colonial living can result in a greater variation in individual experiences which may be one reason for a greater variation in individual behavioural tendencies (Pruitt et al. 2012). Interestingly, there is evidence for social phenotype affecting behavioural type in social spiders, such that relatively social individuals show lower activity levels and less aggressive

behaviour towards predatory cues than those individuals that are relatively asocial (Pruitt et al. 2008). Indeed, social spiders more generally exhibit individual personalities which suggest that in these monomorphic societies this could be one way of maintaining task differentiation between individuals (Grinsted et al. 2013; Grinsted and Bacon 2014).

One group that is ideal for investigating the role and evolution of personalities with social complexity are the queenless ponerine ants. Unlike most ants, ca. 100 species of ponerine ants have secondarily lost their queen caste and reverted to a simple societies in which all females can mate and potentially become the reproductive individual (Peeters 1991; Peeters 1997). In the monomorphic dinosaur ant *Dinoponera quadriceps*, reproduction is monopolised by a single, mated gamergate worker, the ‘alpha’, who enforces her dominance over her subordinates directly with aggressive behaviours and indirectly using pheromone cues (Monnin and Peeters 1998). The evolutionary reversion from complex societies (a state which we know exhibits strong personalities and behavioural syndromes at multiple levels; Jandt et al., 2014) to simple societies, makes queenless ponerines such as *Dinoponera*, a potentially valuable study system for investigating the evolution and implications of personalities in social animals. Here we investigate whether dinosaurs ant show personalities or behavioural syndromes, and if these are related to social position within the reproductive hierarchy.

9.3 Methods

9.3.1 Study organisms

We used 11 colonies of *D. quadricaps*, which were collected at Campo Formoso, Bahia, Brazil in November 2014. All colonies were maintained in the lab at 27°C and 80% relative humidity for at least six months prior to the experiment. Colonies were fed on *Tenebrio* larvae and apple daily and had *ad libitum* access to water. Each individual was uniquely marked using numbered discs (EH Thorne Ltd.) glued on their pronotum.

9.3.2 Determination of the dominance hierarchy

Firstly, to determine the dominance hierarchy in each colony, the behaviours and locations of all workers in each colony were recorded daily for two weeks. Medium and high ranking ants in *D. quadricaps* tend to be associated with brood (Monnin and Peeters 1998; Monnin and Peeters 1999; Asher et al. 2013), so any individual observed on at least one occasion either with, or on, brood was identified as a potential medium or high-ranker. The precise hierarchical positions of these individuals were then determined in isolated dyadic interaction assays. This method pairs every combination of ants studied, with observation of ritualised dominance behaviours between the pair then revealing which individual is the dominant or submissive individual in each dyadic interaction. This has previously shown to be a reliable and robust way to establish dominance hierarchies in this species (Grainger et al. 2014). Individuals were taken from their colonies, placed individually in pots (85 mm x 75 mm x 55 mm) and allowed to acclimatise for 15 min. Pairs of ants were then re-introduced into a new pot, their behavioural interaction observed and, where possible, the dominant ant recorded. Dominant *D. quadricaps* ants stand tall with their antennae pointed forward and placed either side of the subordinate individual, which adopts a flatter body posture with antennae laid flat on their head and directed posteriorly (Monnin and Peeters 1998; Monnin and Peeters 1999; Grainger et al. 2014). This reaction normally occurs within

the first 60 s of contact between ants. We then ranked individuals based on the number of interactions in which they were dominant. We avoided using the highest ranked individual (the alpha) and selected for further study individuals of medium to high rank, split approximately equally from each of the 11 colonies to give 33 ants in total.

9.3.3 Behavioural observations

Behavioural observations were made for the next four days for each of the focal ants. Within the colony, we recorded ‘sociability’ (defined as the number of ants within 5 cm of the focal ant), as well as if the focal ant was undertaking brood care behaviours (carrying, licking or antennating larvae or eggs). Focal ants were then isolated and a number of individual-level assays were carried out. We recorded activity level, phototaxis and aggression, specific assays that have been shown to separate out behavioural castes in other species (Pamminger et al. 2014; Norman and Hughes 2016). We predicted that high rankers would in general show lower activity levels (as higher rankers perform fewer tasks than lower rankers; Monnin et al., 2003), lower phototaxis (as they tend to remain in the nest and away from any ‘risky’ tasks such as nest defence or foraging; Asher et al., 2013; Nascimento et al., 2012), and higher levels of aggression (known to be associated with higher ranks; Cant et al., 2006; Monnin and Peeters, 1999). To measure activity levels, individuals were placed in a 90 mm Petri dish lined with filter paper, allowed to acclimatise for 2 min and then filmed for 5 min using a Logitech c920 webcam. Activity level was quantified using Antrak path analysis software (Tranter et al. 2014). To measure phototaxis, individuals were placed in a 90 mm Petri dish, lined with filter paper, and half blackened-out with tape. Individuals were left for 2 min to acclimatise, then filmed for 5 min and the proportion of time spent in the light half of the Petri dish recorded. To measure aggression, individuals

were placed in a pot (85 mm x 75 mm x 55 mm), allowed to acclimatise for 5 min and then carefully touched on the head with the tip of a toothpick, similar to Pamminer et al. (2014). The reaction of the ant was scored as an aggressive or non-aggressive interaction (non-aggressive = ignore (0) whereas aggressive interactions counted as gaping mandibles (1) in a threat response, biting (2) or attempting to sting the toothpick (3)).

The above behavioural variables were quantified for each ant, daily for four days. Two weeks after the first set of assays, all ants were assayed a second time in the same way for brood care and ‘sociability’, activity level, phototaxis and aggression. The assays were again repeated daily for four days (giving eight repeated measurements for each ant in total). On the 5th day the isolated dyadic interaction assays were conducted again, using both the focal ants and all ants observed at least once in the last 2 weeks to be located in, or near, the brood to reassess the dominance hierarchies.

9.3.4 Statistical analysis

We used generalised estimating equations (GEE) to test for individual consistency in behaviour, with individual as the subject variable and time point (1-8) as a repeated-measures, within-subject variable. Separate models were constructed for each behavioural variable (activity, phototaxis, aggression, brood care and sociability).

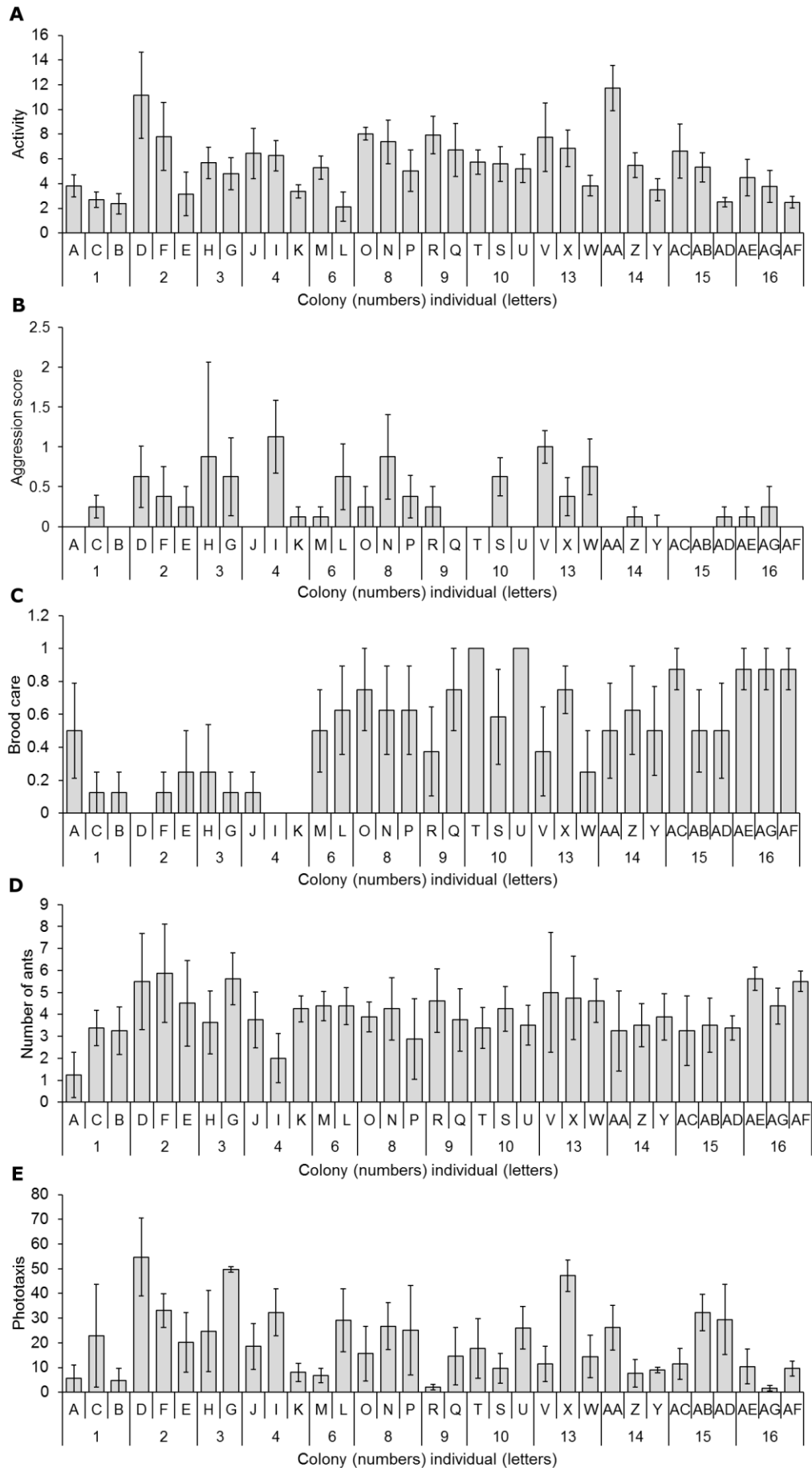
Individual nested within colony was included as a fixed factor (to test if individuals differed in their behaviours), as well as time point (to test if individuals were consistent in their behaviours), colony-of-origin (to control for our individuals being sampled from different colonies), and rank in the hierarchy (to see if rank in the hierarchy showed an association with behavioural traits). To examine whether there were behavioural syndromes we calculated the mean of the eight repeated measures for each individual

and used Spearman's rank correlation to determine whether there were significant relationships between the different behavioural variables across individuals. Multiple testing was adjusted for using the false discovery rate (FDR) method (Benjamini and Hochberg 1995).

9.4 Results

Individuals showed consistency across time in their activity level, aggression, brood care and sociability, with no significant effects of the time repeated-measures on these variables ($\chi^2 = 7.07$, $P = 0.42$, $\chi^2 = 10.1$, $P = 0.18$, $\chi^2 = 9.6$, $P = 0.21$, and $\chi^2 = 6.65$, $P = 0.47$, respectively, $df = 7$ in all cases; Figure 9.2). Individuals were not consistent across time their phototactic behaviour ($\chi^2 = 71.2$, $df = 7$, $P < 0.001$) with phototaxis levels generally dropping markedly between the two behavioural observation period.

Figure 9.2 Behavioural consistency in dinosaur ants (*overleaf*). Mean \pm s.e. from eight time points (A) activity level (outputted from Antrak custom tracking software), (B) aggression score (aggression to a threat stimuli scored 0-3), (C) brood care (instances where individuals were observed tending to brood within the colony), (D) sociability (number of ants within 5cm of the focal ant within the colony), (E) phototaxis (percentage of time spent in the light half of a Petri dish), for 33 high or medium ranking *Dinoponera quadricaps* dinosaur ants from 11 colonies.



There were consistent differences between individuals in activity level and aggression ($\chi^2 = 65.2$, $P < 0.001$; $\chi^2 = 71.2$, $P < 0.001$, $df = 24$ in both cases) Generally these two assays showed relatively high variation between individuals, particularly aggression, with many individuals showing no aggressive responses at all (Figure 9.2). Ants also showed differences between individuals in phototactic behaviour ($\chi^2 = 64.2$, $df = 24$, $P < 0.001$), but these were not consistent over time. We observed no consistent differences between individuals in either brood care or sociability ($\chi^2 = 25.6$, $P = 0.37$; $\chi^2 = 24.3$, $P = 0.45$, $df = 24$ in both cases).

Rank was significantly associated with phototaxis, aggression and brood care ($\chi^2 = 22.4$, $P = 0.004$; $\chi^2 = 32.1$, $P < 0.001$; $\chi^2 = 16.6$, $P = 0.035$, $df = 8$ in all cases), but showed no relationship with either activity level or sociability ($\chi^2 = 6.5$, $P = 0.59$; $\chi^2 = 9.31$, $P = 0.32$, $df = 8$ in both cases). In general, lower rankers were generally more phototactic and aggressive, but showed slightly less brood care tendency than higher rankers (Figure 9.3). Colony-of-origin had a significant effect on all behavioural variables: activity, phototaxis, aggression, brood care and sociability ($\chi^2 = 42.6$, $P < 0.001$; $\chi^2 = 53.7$, $P < 0.001$; $\chi^2 = 71.8$, $P < 0.001$; $\chi^2 = 28.7$, $P = 0.002$; $\chi^2 = 76.2$, $P < 0.001$, $df = 11$ for all cases).

There were no significant correlations between the five behavioural variables measured (activity, aggression, brood care, sociability, phototaxis) once we had corrected for repeated testing (Table 9.1).

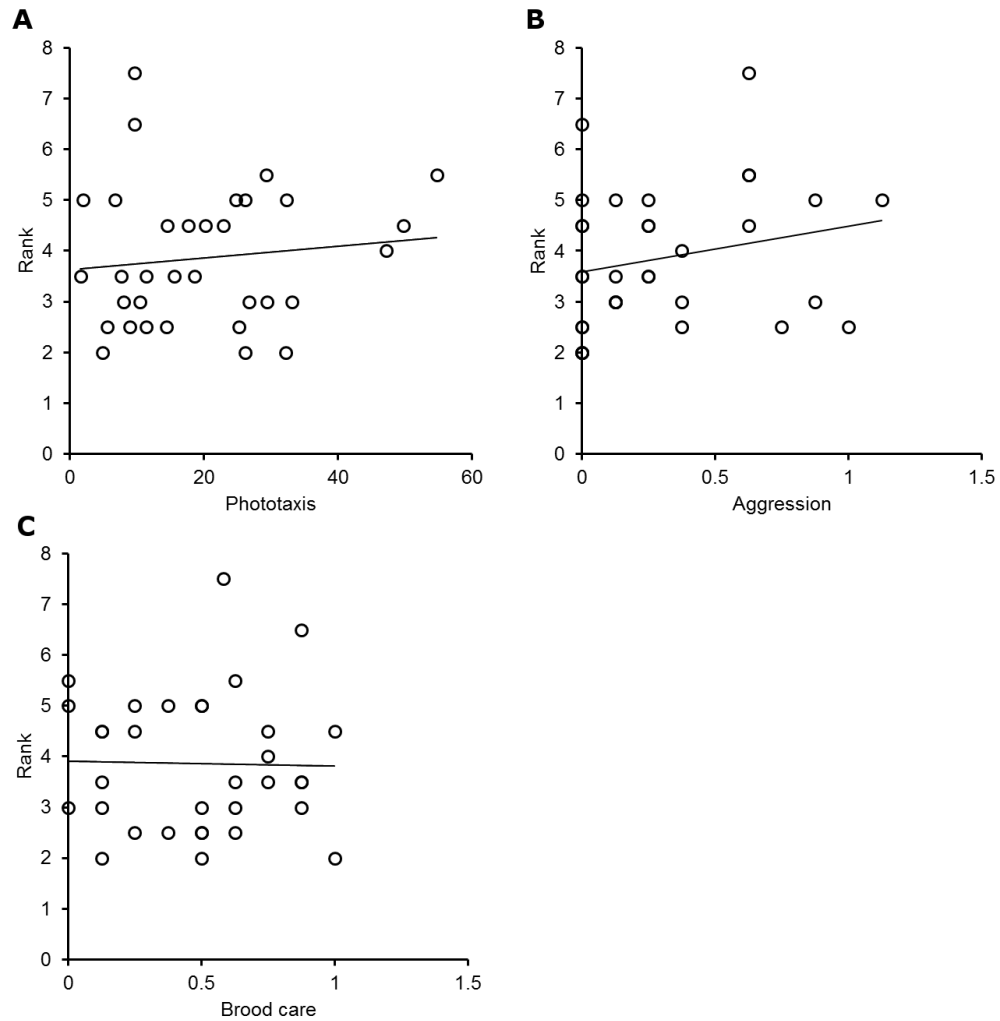


Figure 9.3 Associations between rank and behaviour in dinosaur ants. Relationships between social dominance rank and mean (A) phototaxis (percentage of time spent in the light half of a half blackened Petri dish), (B) aggression score (aggression to a threat stimuli scored 0-3), (C) brood care (instances where individuals were observed tending to brood within the colony) for 33 high or medium ranking *Dinoponera quadriceps* dinosaur ants from 11 colonies.

9.5 Discussion

Individual *D. quadriceps* dinosaur ants were consistent over time for four of the five behavioural variables we measured (activity level, aggression, brood care and sociability), and showed significant differences between individuals of different rank for two of these, activity level and aggression. Phototaxis, aggression and brood care were

Table 9.1: Correlation coefficients for five behavioural variables measured from 33 ants from 11 colonies. P values have been corrected for multiple testing using the FDR method (Benjamini and Hochberg 1995).

Behaviour	Statistic	Activity	Phototaxis	Brood care	Aggression	Sociability
Activity	r_s	.	0.275	0.004	0.195	0.092
	P-value	.	0.31	0.98	0.46	0.79
Phototaxis	r_s		.	-0.199	0.362	0.67
	P-value		.	0.46	0.15	0.79
Brood care	r_s			.	-0.35	-0.069
	P-value			.	0.15	0.79
Aggression	r_s				.	0.385
	P-value				.	0.15
Sociability	r_s					.
	P-value					.

correlated with social dominance rank, with higher ranked ants being less aggressive and phototactic, and showing more brood care. There was no evidence of behavioural syndromes for the variables we measured.

The results provide evidence for consistent individual differences in behaviour, i.e. personalities, within dinosaur ants, even among the subset of relatively high-ranking individuals studied here. The evolution of personality first depends upon individuals differing in their behavioural type, so called ‘behavioural niches’ (Bergmüller and Taborsky 2010). Given the existence of personalities across ants and bees with complex societies as well as behavioural types/syndromes existing in some wasp societies (Turillazzi and West-Eberhard 1996; Reeve and Nonacs 1997; Giray et al. 2000; Cant et al. 2006; Wray et al. 2011; Scharf et al. 2012; Pinter-Wollman et al. 2012; Keiser et al. 2014; Monceau et al. 2014), the retention of personalities in dinosaur ants after their reversion to simple societies, suggest that personalities are probably ancestral in social insects. It is possible that individual differences in behaviour may have laid the

evolutionary groundwork for the division of labour within insect societies that has been key to their ecological and evolutionary success.

Interestingly, despite strong evidence for behavioural syndromes or trait associations in other ants, both at the individual and colony levels (Chapman et al. 2011; Scharf et al. 2012; Bengtson and Dornhaus 2014), we did not find any evidence for behavioural syndromes within dinosaur ants. Given the ubiquity of behavioural syndromes across the animal kingdom (Sih et al. 2004a), it would be surprising if they were not present in dinosaur ants. One possible explanation could be that personalities and behavioural syndromes are hierarchical with the latter potentially being lost first upon dinosaur ants evolutionary reversal to its current, primitively eusocial state. Alternatively, there may be multiple combinations of traits which are associated with high rank and therefore different pathways to social success (Norman et al. 2016). It is also possible that the assays we used were not optimum for detecting behavioural syndromes or possibly that the relatively high-ranking individuals we restricted our study to were behaviourally constrained by their role within the hierarchy. The lack, or presence, of behavioural syndromes, and their strength, is likely important to social organisation, so further investigation of this, including in lower ranked individuals, would be worthwhile.

We also observed a significant relationship with rank in the hierarchy for a number of our behavioural variables, with high rankers being less phototactic and aggressive and more likely to be found with brood than lower rankers. This is in keeping with previous work carried out on this species, which reported higher rankers undertaking fewer ‘risky’ behaviours (Monnin and Peeters 1999; Asher et al. 2013). This suggests that the hierarchy is likely one of the most important factors in task allocation within this species, given that within this system all workers can reproduce

and therefore can maximise their probability of achieving direct fitness by being as high up the hierarchy as possible (Monnin and Peeters 1998; Monnin and Peeters 1999; Monnin et al. 2003).

Further work is needed to understand the organisation, and proximate causes generating, personalities in eusocial species with simple societies as well as solitary Hymenoptera. Understanding the relationship between social structure and personality is of particular importance to glean further insight in to the behavioural processes surrounding the evolution of eusociality.

10 General Discussion

In this thesis I explore the proximate and ultimate causes of worker and reproductive division of labour in ant societies. This work falls under four main themes: 1) the pleiotropic role of juvenile hormone in worker and reproductive division of labour, 2) threat – or defensive – behaviours and factors affecting these behaviours at multiple levels of social organisation, 3) behavioural trait combinations – so called ‘behavioural syndromes’ – or lack thereof in ant societies, and finally 4) the genetic influences on caste, which can sometimes outweigh environmental processes in deciding reproductive potential in certain systems.

Much of the work surrounding the proximate underpinnings of the division of labour has focused on the role of the endocrine system. This makes hormones prime candidates for the proximate mechanisms controlling both morphological and behavioural castes across social insects (Nijhout 1998; Bloch et al. 2009). Juvenile hormone (JH) occupies such a central, regulatory role within the social insects: it physiologically integrates environmental information and can subsequently affect both reproduction and behaviour simultaneously (Bloch et al. 2000; Hartfelder 2000; Sullivan et al. 2000; Miura et al. 2003; Bloch et al. 2009; Amsalem and Malka 2014). In this thesis I aimed to extend our current knowledge of the behavioural effects of juvenile hormone in ants, as well as look at the role it plays in the regulation and maintenance of dominance societies in a primitively eusocial ant to study the pleiotropic role that this hormone plays in shaping social insect societies.

A large proportion of the evidence for the behavioural effects of juvenile hormone comes from the model system of honey bees. Juvenile hormone has been implicated as a behavioural regulator of age-related division of labour in this model species (Robinson 1987a; Sullivan et al. 2000; Schulz et al. 2002), and there is correlational evidence of similar effects in some ant species (Giray et al. 2005; Lengyel et al. 2007; Shorter and Tibbetts 2008; Penick et al. 2011; Dolezal et al. 2012). However, we did not previously have direct, experimental evidence from ant societies, which evolved eusociality independently from bees. In Chapter 2 I give, to my knowledge, the first direct experimental evidence for the effect of juvenile hormone on worker division of labour in ant societies, as well as the behavioural mechanisms by which this effect is produced. This work provides evidence for a ‘toolkit’ style situation, indicating that this regulation of behaviour by juvenile hormone is either ancestral in Hymenoptera or has evolved multiple times. Interestingly, however, in Chapter 3 we see no effect at all of juvenile hormone on worker behaviour in the primitively eusocial ant *Dinoponera quadricaps*. This species is of particular interest from an evolutionary perspective because it is one of ~100 species of ponerine ants that have secondarily lost the queen caste, and instead evolved a gamergate system in which colonies exhibit a dominance hierarchy headed by a single, mated, dominant gamergate worker (Peeters 1997). Interestingly, similar observations have been made in *Ectatomma alphas*, an ant genus with a similar social structure (Cuvillier-Hot et al. 2004b), suggesting that, perhaps, in gamergate societies a lack of behavioural regulatory function of JH may be associated with their evolutionary reversal to a more primitive state. Despite JH not showing an effect on worker behaviour in *Dinoponera*, we did observe a clear effect of our hormone treatment on fertility and subsequent reproductive potential in these ants (Chapter 3). Instead of observing the gonadotropic effects that JH shows in solitary

insects, we see the classical reversal of this role that is observed in many eusocial insect reproductives (Robinson and Vargo 1997; Azevedo et al. 2011; Pamminer et al. 2016), suggesting a lack of causation between the remodelling of JH structure and the level of social complexity observed in eusocial societies.

A key component to the survival of social insect societies is to be able to accurately detect, respond, and communicate these threats to other members of the colony. In this thesis I studied proximate and ultimate determinants of defensive behaviour at multiple levels in ant societies: individual-level propensity to detect threats (Chapter 4), species-level comparisons of alarm communication (Chapter 5) and colony-level investments in both defensive and behavioural phenotypes following continued threat disturbance (Chapter 6).

Both in Chapters 4 and 5 I looked at factors affecting individual-level propensity to detect threats, either from nestmates and non-nestmates or from constituents of the alarm pheromone. In the former we show age and previous experience, rather than morphological caste, is important in correctly identifying threats. This pattern also holds true in Chapter 5 when individual ants are assayed for detecting specific stimuli from alarm pheromone – older ants respond more readily than younger ants, whereas morphological caste had no effect on worker propensity to respond to alarm pheromone. Age polyethism is the most common method of dividing labour among the workforce in bees, ants and wasps: individuals start their lives with internal tasks such as brood care or within-nest work and as they age switch to more ‘risky’, external tasks such as foraging or nest defence (Seeley 1982; Morel et al. 1988; Jaisson 1991; Wakano 1998; Camargo et al. 2007; Muscedere and Traniello 2012). While we know that individuals

make these behavioural repertoire shifts, these chapters show that individual behavioural traits also alter to match such age-related tasks where being able to be more threat responsive would be advantageous for these external tasks such as foraging, or nest defence.

While we show morphological caste may not affect an individual's recognition of a threat (either from a conspecific, or from constituents of alarm pheromone), this does not necessarily mean that the responses of the different castes once the threat has been detected will be the same. Indeed, often larger bodied individuals are the ones to respond most aggressively to threats (Nowbahari et al. 1999; Huang 2010; Hölldobler and Wilson 2010). Interestingly, however, within the phenotypically plastic leaf-cutting ant species *Atta colombica*, repeated long-term exposure to threat disturbance did not cause colonies to preferentially invest in to larger bodied individuals, rather it altered colony-level behavioural phenotypes in as little as 2 weeks (Chapter 6). Indeed, this chapter gives evidence for all castes up-regulating not only their threat responsiveness but also aggression in response to repeated threat disturbance, suggesting a colony-level behavioural phenotype (Chapman et al. 2011; Bengston and Dornhaus 2014). Such a dynamic behavioural flexibility could also offer a more adaptable and plastic response to environmental perturbation than a potentially costly investment into a fixed morphological defensive phenotype (Tufto 2000; Sih et al. 2004a).

Lastly, with respect to defensive behaviours, in Chapter 5, we compared the alarm pheromone composition of seven attine species, as well as further behavioural assays in four leaf-cutting ant species to identify how these communities communicate alarm to their nestmates. It is often predicted within the animal kingdom that there should be low interspecific variation in alarm communication across the animal kingdom, as it should benefit species to use other species alarm cues to as to accurately

detect alarm cues which could have dramatic effects on survival if missed (Blum 1969; Hazlett 1994; Vander Meer and Alonso 1998; Commens and Mathis 1999; Laforsch et al. 2006). However, contrary to this prediction we find surprisingly high levels of interspecific difference between species in alarm pheromone composition, even within the genus of *Acromyrmex* we observe differences in the behaviourally active constituent of the alarm pheromone. The exact reason behind this is unclear, although previous work has also found many of these volatiles in attine heads, (Moser et al. 1968; Crewe and Blum 1972; Do Nascimento et al. 1993; Hernandez et al. 1999) as well as there being increasing evidence for alarm pheromone playing a role in intraspecific recognition (Hughes et al. 2001a; Hernández et al. 2002; Francelino et al. 2008). Perhaps mandibular secretions serve purpose other than alarm cues, such as antimicrobial properties (North et al. 1997), or it could be an adaptation to allow interspecific recognition between sympatric species. One consistency, however, is the behaviour of ants towards alarm pheromone. Both in this chapter and Chapter 4 we have used the mandible opening response assay to look at the contribution alarm pheromone plays in threat detection and subsequent nestmate recognition. While harnessed ants certainly respond to alarm pheromone produced by live ants (Chapters 4 and 5) - and indeed the pheromone is hypothesised to play an important role in nestmate recognition (Francelino et al. 2008) – this process is much more effective with the additional cues of cuticular hydrocarbons (Chapter 4).

Within the study of animal behaviour, recent focus has been on the advent of so-called ‘behavioural syndromes’, where individuals show consistent behaviours – ‘behavioural types’, and/or behavioural trait associations across multiple contexts (Sih et al. 2004a; Jandt et al. 2014). These behavioural trait associations in particular are suggested to be a

relatively common phenomenon, and indeed, associations are often observed in many animal species between traits such as aggression and boldness or activity level (Huntingford 1976; Coleman and Wilson 1998; Sih et al. 2004b). Interestingly, however, many of these syndromes can be maladaptive if expressed either inappropriately or at the wrong time (Wilson and Boelkins 1970; Arnqvist and Henriksson 1997; Ritters et al. 2000; Sih et al. 2003; Viñuela et al. 2010). We provide evidence for some behavioural trait associations in *Lasius* queens and their association with productivity in Chapter 8, as well as a lack of evidence for such trait associations in the primitively eusocial dinosaur ants in Chapter 9. It is interesting that we do not observe any significant behavioural syndromes in *Dinoponera*, although very little work to date has been carried out on either behavioural syndromes or personality in primitively eusocial species. There is some evidence for behavioural syndromes scaling with social complexity in spiders (Pruitt et al. 2008), although the exact relationship between social complexity and behavioural syndromes is currently lacking (Jandt et al. 2014) and, indeed, it is hard to speculate without a broader comparative dataset.

Finally in this thesis, we show evidence for genetic caste determination in *Messor* harvester ants (Chapter 7). Unlike the previous chapters, which have focussed primarily on the environmental determinants of caste and task we show genetic evidence for an unorthodox mating system in the Iberian population of *Messor barbarus*. Here two distinct, cryptic genetic lineages must mate to form heterozygous workers and where gynes (sexual females) are produced as a result of pure lineage mating. This socially hybridogenetic system, whilst rare is also observed in five other ant genera: *Cataglyphis* (Eyer et al. 2013; Darras et al. 2014b; Darras et al. 2014a), *Pogonomyrmex* harvester ants (an unrelated genus) (Helms Cahan et al. 2002; Helms Cahan and Keller 2003;

Schwander et al. 2007) and *Wasmannia*, *Paratrechina* and *Vollenhovia emeryi* (Fournier et al. 2005; Ohkawara et al. 2006; Pearcy et al. 2011), although there are distinct differences between systems observed, most notably, in male and queen production (see Figure 10.1). Indeed, to date, these are the only five (now six) ant genera to show a strict genetic caste determination (GCD), across two sub-families whereas in almost all other eusocial hymenoptera caste determination appears to fit somewhere between purely environmental caste determination (ECD) and GCD (Anderson 2008; Schwander et al. 2010).

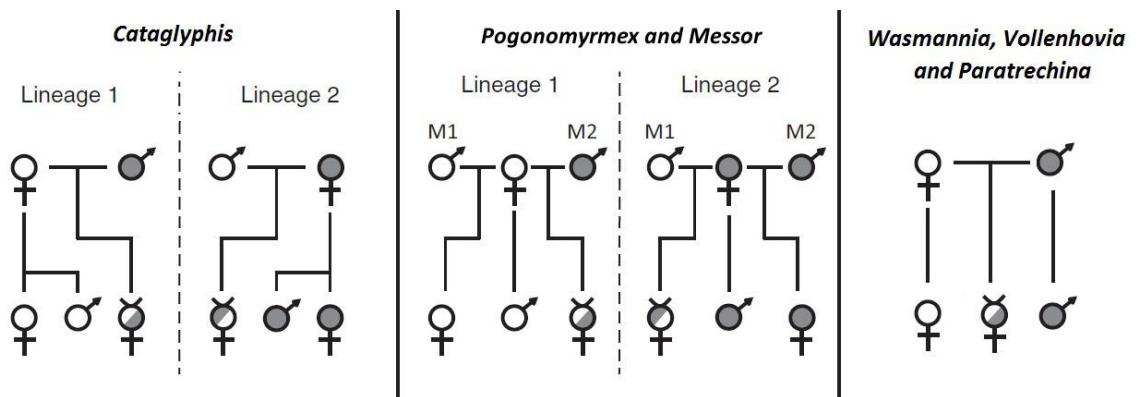


Figure 10.1 Social hybridogenetic systems in ants. All three dependent lineage systems known to date within ant societies detailing how each system produces workers, new queens and males. Social hybridogenesis in *Messor* ants mirrors the system also observed within *Pogonomyrmex* harvester ants (unrelated genus).

The origins of this system, however, still remain unclear. There are a number of different hypotheses regarding the origins of such a system. The first suggests that there could be just one locus is responsible for such caste determination (therefore heterozygous individuals at this locus become workers and homozygotes queens) (Volny and Gordon 2002). In *Pogonomyrmex* one hypothesis is that the linages arose from a recent hybridisation between the ECD systems of *P.barbatus* and *P.rugosus*, such that the resulting GCD is a product of the incompatibilities between the two nuclear loci (Helms Cahan and Keller 2003). This model also goes a way to explain the rare occurrence of heterozygous queens, where double heterozygotes retain bi-potency

but will almost only ever produce workers as too many pure lineage queens are present in the colonies, monopolising resources or otherwise inhibit heterozygous individuals from developing in to queens (Helms Cahan and Keller 2003; Anderson 2008). The third hypothesis suggests that hybridisation was not the original cause of such GCD (merely the mechanism by which it can spread between species) whereas the causal agent of the GCD itself surrounds the premise of a genetic mutation with a major effect on caste determination, generating genetic conflict and resulting in the partitioning and maintenance of distinct sets of alleles which confer differential development in castes (Anderson et al. 2006). The final hypothesis is based on the assumption that the two lineages originally diverged because of the interaction between cytoplasmic and nuclear genes, such that individuals with ‘matching’ nuclear and mitochondrial DNA (within-lineage offspring) are biased for queen development whereas in matings arising from between lineages, mis-matched nuclear and cytoplasmic DNA results in worker development (Linksvayer et al. 2006). Interestingly, we find two instances of cyto-nuclear mismatched (mtDNA from one lineage and nDNA from the other) within queens in Chapter 7, suggesting that rare introgression events may exist. It is suggested that these introgressions could enable the dual evolution of both nuclear and mitochondrial genomes to maintain optimal functions in hybrids as incompatibilities between these two genomes can reduce hybrid fitness (Darras and Aron 2015).

Furthermore, GCD has been proposed to originate because of conflict between queens and developing larvae over their caste fate (Strassmann and Queller 2008) therefore is also possible that such ‘hard-wired’ GCD may be a more common phenomenon than previously thought, given taxonomic research bias as well as the advent of more efficient techniques regarding molecular detection of such systems (Keller 2007).

Broadly speaking, this work has aimed to demonstrate the genetic and environmental processes shaping division of labour outside of the classical model system of honey bees. Of course, much is left to study, particularly with respect to elucidating the role of juvenile hormone within insect societies. Given its largely pleiotropic nature across organisms of varying social complexity it is likely that this hormone, or at least its regulatory architecture, has been remodelled convergently with shifts in social lifestyle and offers a potential physiological mechanism for integrating genetic and environmental stimuli with behaviour and development. A second, worthwhile area for future study would be the active search for other systems where genetic influences on caste may outweigh environmental cues. Advances in molecular biology make these discoveries much more feasible, such that these phenomena may be found more easily in non-model species. Finally, future, comparative work should aim to study societies of varying social complexity outside of this model system, enabling us to better understand the patterns and processes influencing both behaviour and phenotype within the animal kingdom.



Figure 10.2 Dinosaur ant hunters. The author and fellow lab members collecting dinosaur ants from Bahia, Brazil, November 2014

11 References

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12 Supplementary Figures

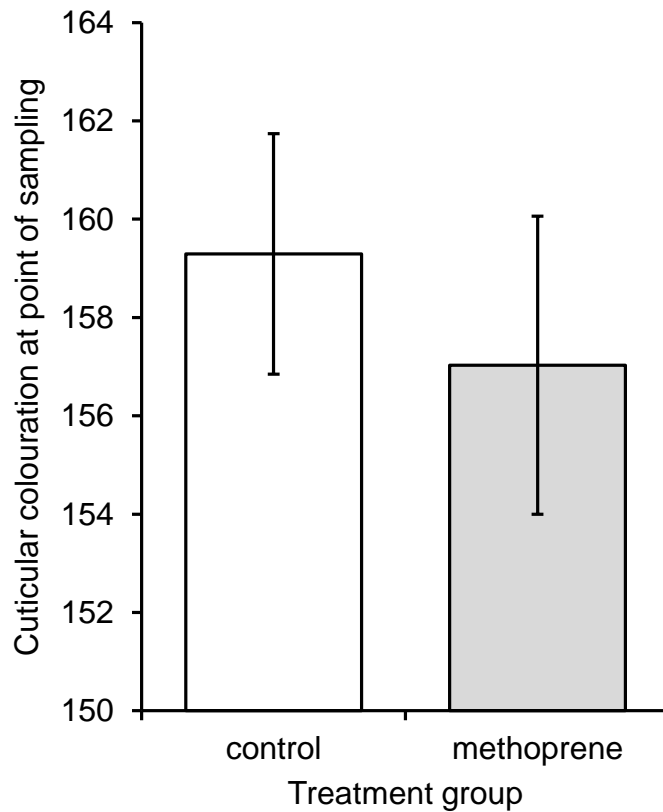


Figure S2.1: For comparison with the effects of juvenile hormone manipulation, we determined the cuticular colouration of leaf-cutting ant within-nest workers randomly assigned to either a solvent control, or methoprene treatment (N= 41 for control, N = 37 for JH sampled evenly from five colonies of *Acromyrmex octospinosus* leaf-cutting ants). Ants were photographed dorsally and the colouration of the middle third of the rear femur quantified using ImageJ software as per (Armitage and Boomsma 2010). Treatment and control ants did not differ in their cuticular colouration or therefore age ($F_{1,76} = 0.275$; $P = 0.601$).

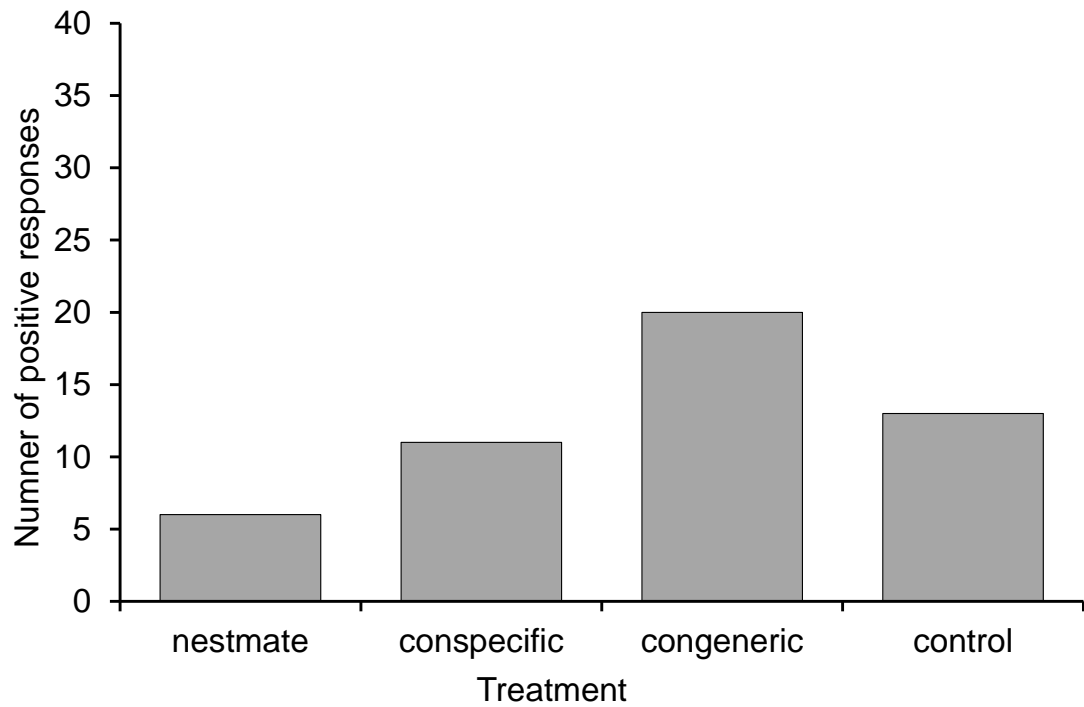


Figure S4.1 The effect of confirmation bias on the MOR assay. The total counts from 40 ants from two colonies that showed a mandible opening response to a nestmate, non-nestmate of the same species, a different species and a control during a double blind trial. Stimuli ants were presented as dead ants that contacted the antennae with their colony origin unknown to the observer at the time of the assay, with their origin unknown to the observer during the experiment. Results from the blind trial did not differ significantly from the non-blind trial ($F_{1, 279} = 0.148$, $P = 0.701$), confirming that the results were not due to observer bias. Focal ants were least aggressive to nestmate ants, showing increasing threat responses to increasingly foreign threats.

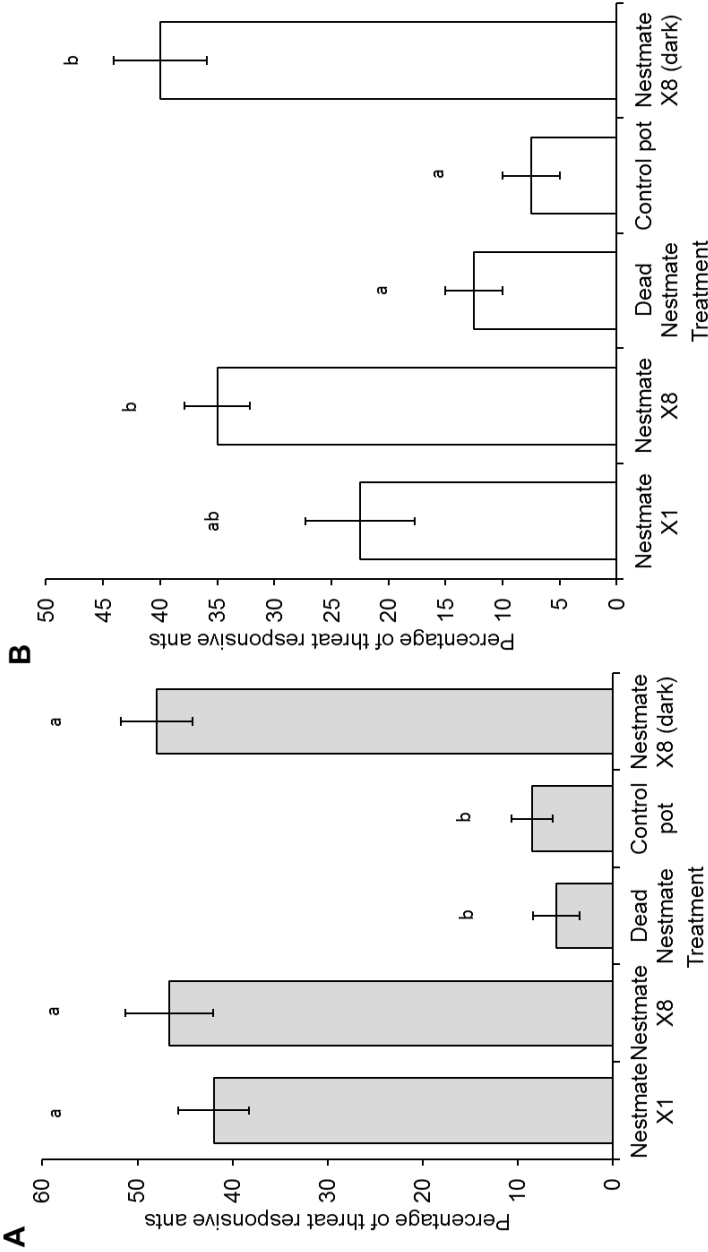


Figure S5.1 The behavioural responses of both a) *Acromyrmex octospinosus* and b) *Acromyrmex echinator* to live alarming nestmates. Mean \pm s.e. percentage of positive mandible opening responses (MORs) to live ants emitting alarm pheromone for either 1 nestmate, 8 nestmates, their respective controls as well as one of the treatments under red light to confirm focal ants were responding to the volatiles emitted by alarming ants rather than the sight of alarming nestmates. Different letters above columns indicate treatments that differed significantly from one another at $P < 0.05$ in pairwise comparisons.

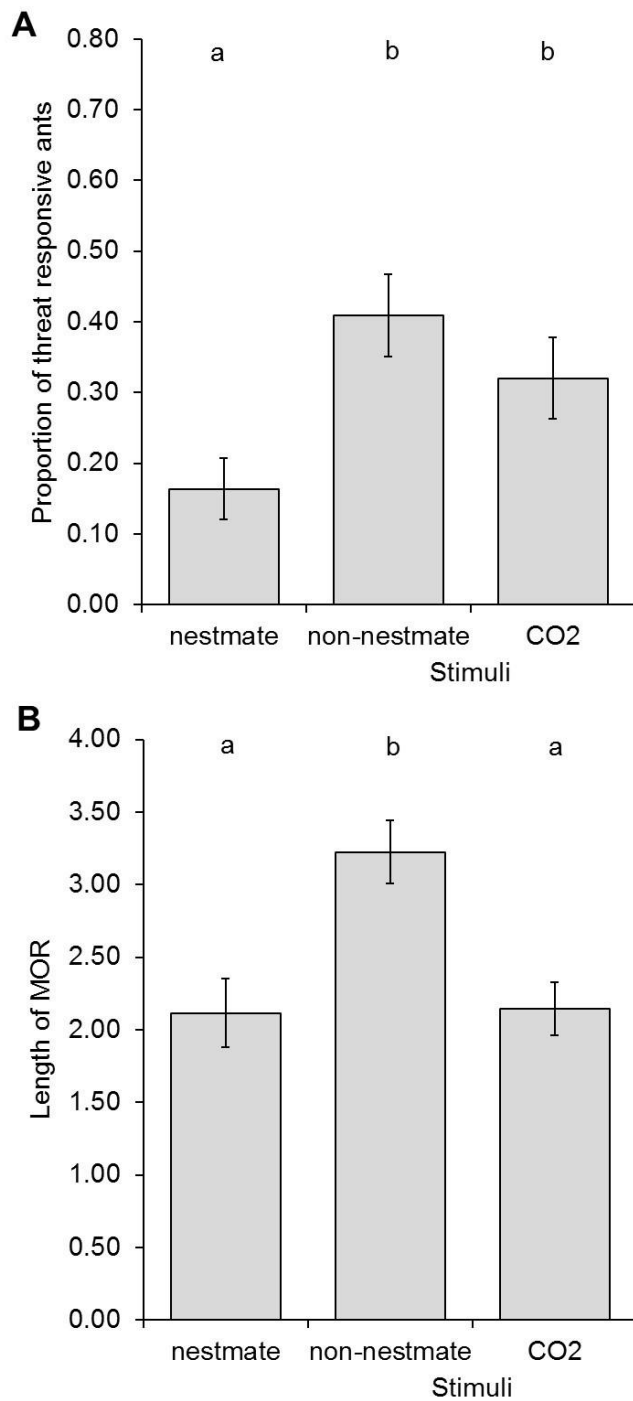


Figure S6.1 Mean \pm s.e A) proportion of positive mandible opening responses (MORs) to four different threat stimuli for all 260 ants from both long term (17 month) disturbed and undisturbed colonies, B) length of MOR to four different threat stimuli for all ants. The latter treatment was designed to simulate a vertebrate predation threat, which should be a specific stimulus for defensive workers to respond to. Different letters above columns indicate significant differences between MOR stimuli at $P < 0.05$ based on pairwise comparisons following GLMM analysis with P values adjusted using the sequential Bonferonni method.

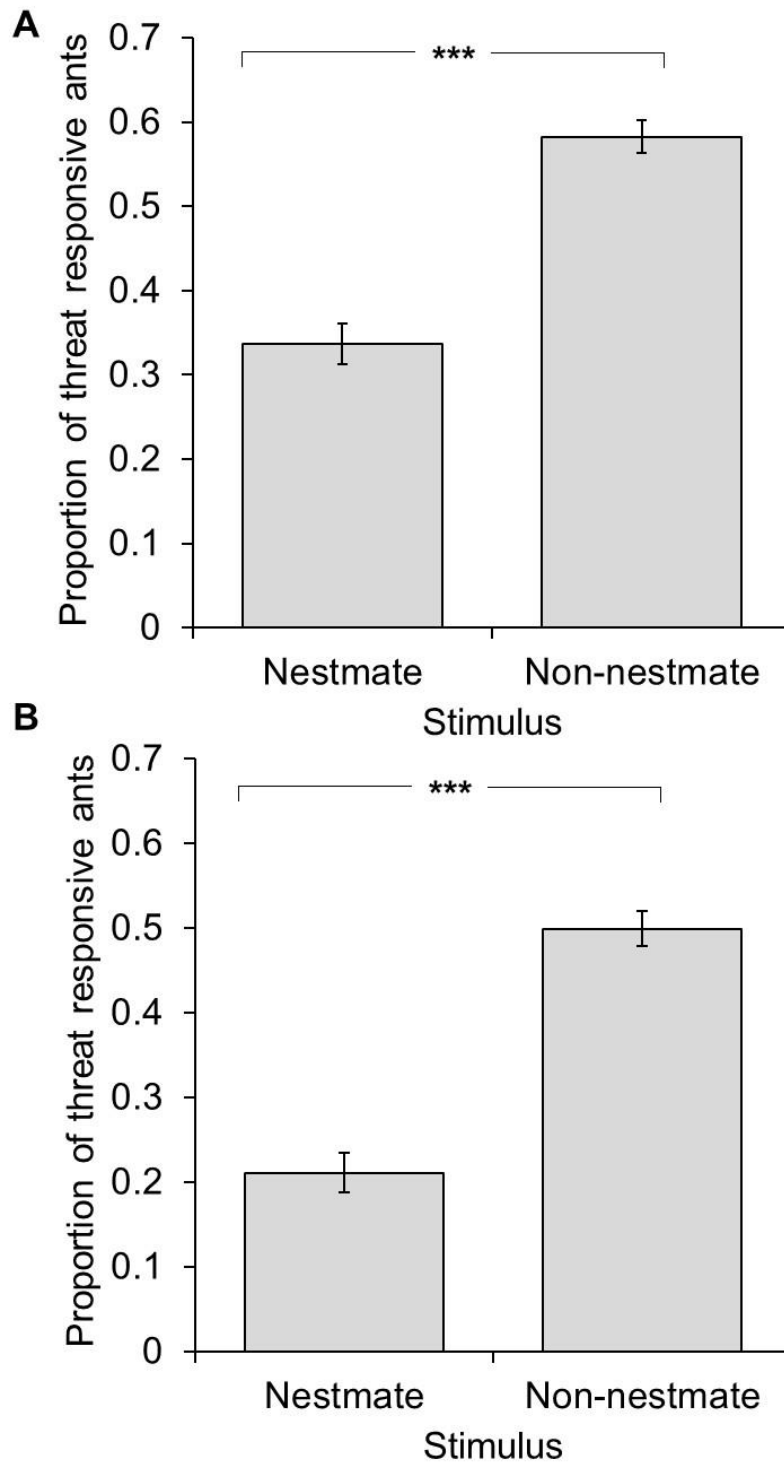


Figure S6.2 Mean \pm s.e A) proportion of positive mandible opening responses (MORs) to two different threat stimuli for all 252 ants from both short term (3 week) threat disturbed and control sub-colonies, and B) proportion of positive mandible opening responses (MORs) to two different stimuli for 252 ants from both threat disturbed and control sub-colonies two weeks after the end of the short term disturbance treatment. Asterisks indicate significant differences between MOR stimuli (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

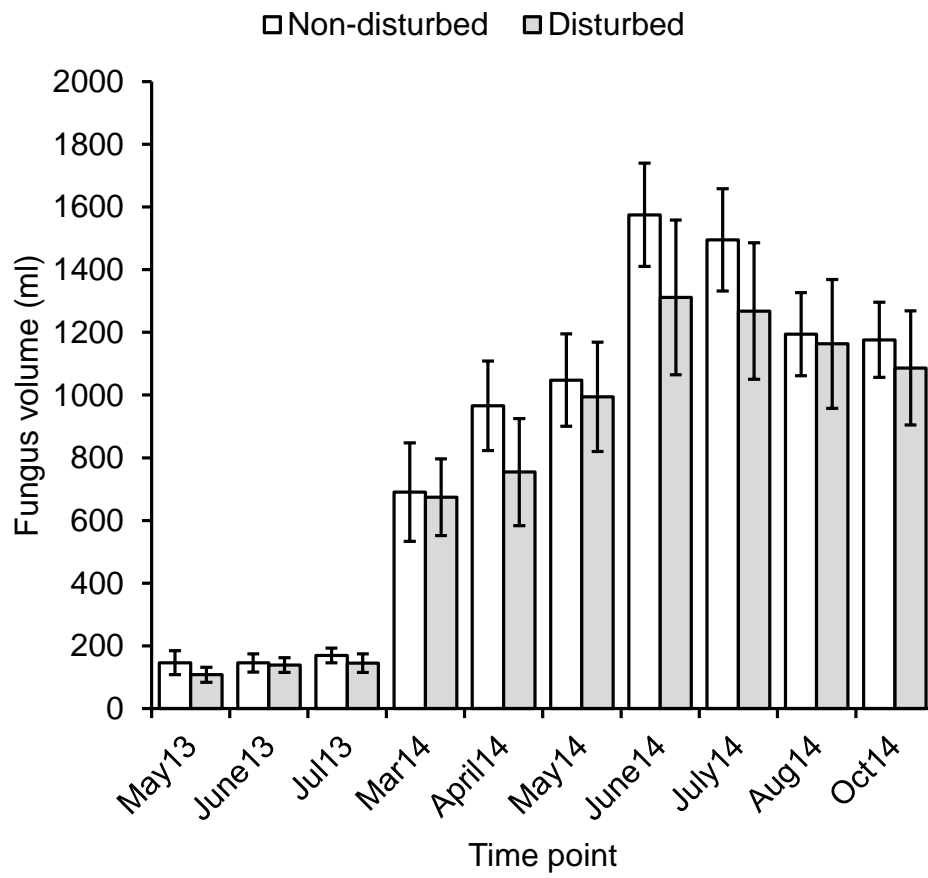


Figure S6.3 Mean \pm s.e. sizes (ml of fungus garden) over a 17 month period of leaf-cutting ant colonies that were either disturbed or undisturbed. The sizes of disturbed and undisturbed colonies did not differ significantly over the course of the experiment (repeated measures GLM: $F_{1,131} = 0.880$, $P = 0.350$)

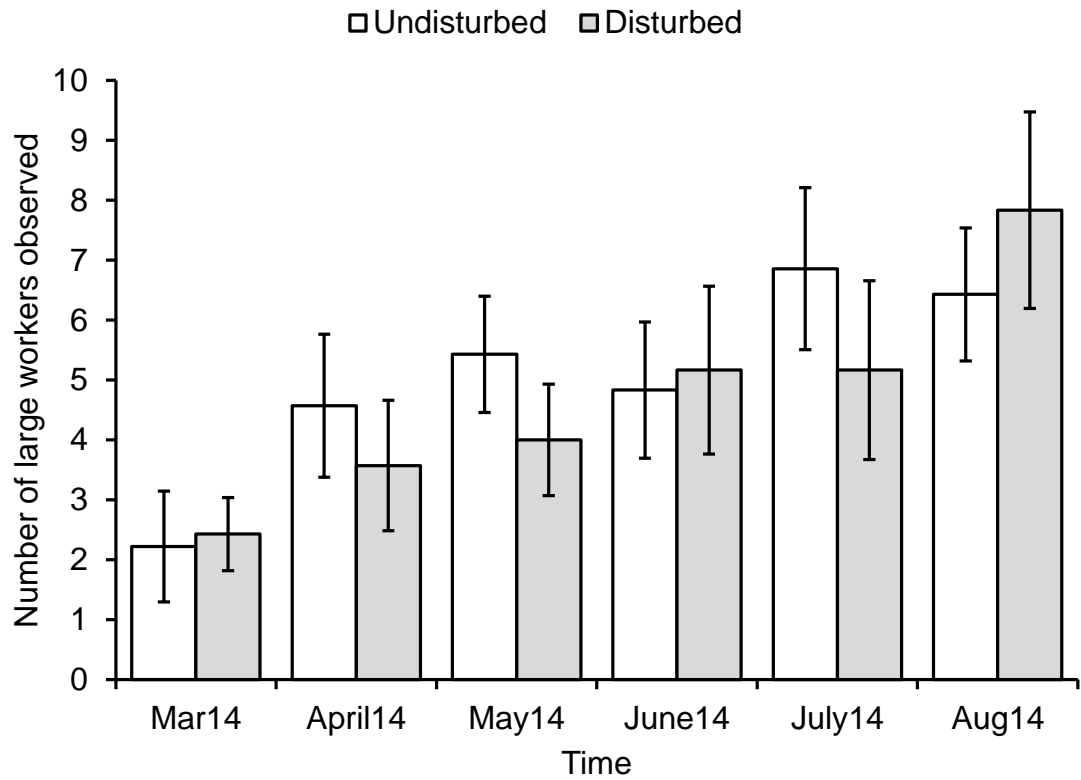


Figure S6.4 Mean \pm s.e counts of large workers observed during a 17 month period where leaf-cutting ant colonies were either disturbed or undisturbed once colonies started to produce larger workers. The number of large workers produced over this time period did not differ significantly between disturbed and undisturbed colonies (repeated measures GLM $F_{1,79} = 0.596$; $P = 0.442$).

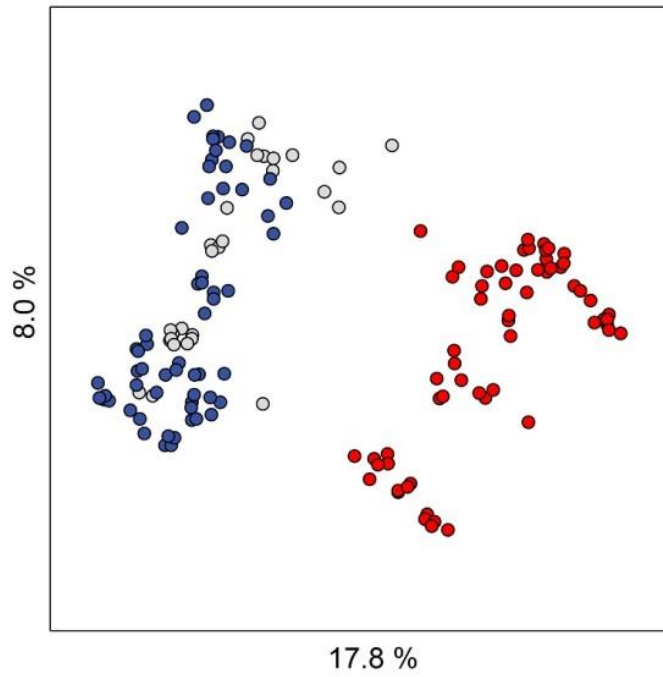


Figure S8.1 PCoA plot based on the genotypes of 158 reproductive individuals (inferred parents of colonies, and queens and males collected during nuptial flights). The percentage of variation explained by each PCoA axis is indicated. *Mbar1*: red, *Mbar2*: blue, unknown: grey (missing information for *Ms2c*).

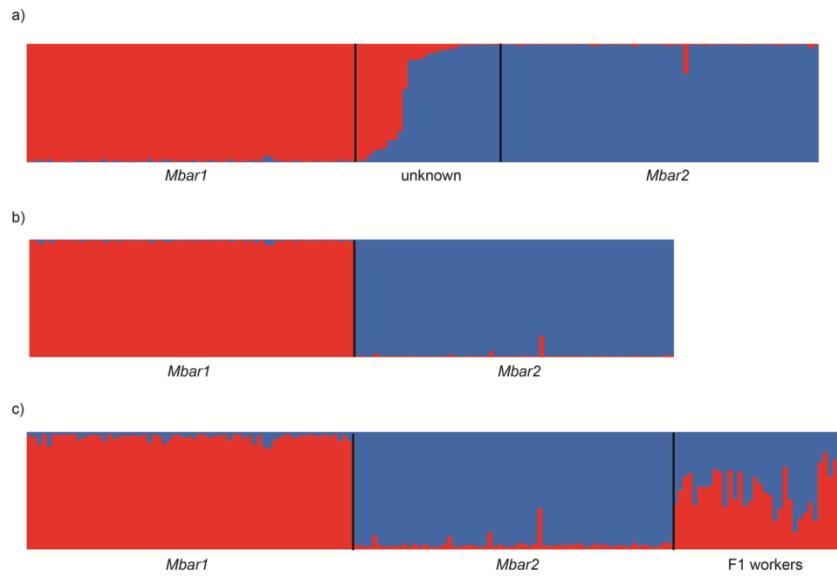


Figure S8.2. Structure bar plot showing the assignment probabilities for $K = 2$ of: a) the 158 reproductive individuals genotyped, including 29 individuals that could not be assigned to a lineage due to ambiguous information at Ms2c; b) the 129 reproductive individuals assigned to a lineage, c) the 129 reproductive individuals assigned to a lineage and also genotypes for 36 workers (one from each colony sampled). For each plot, the highest probability run is shown.

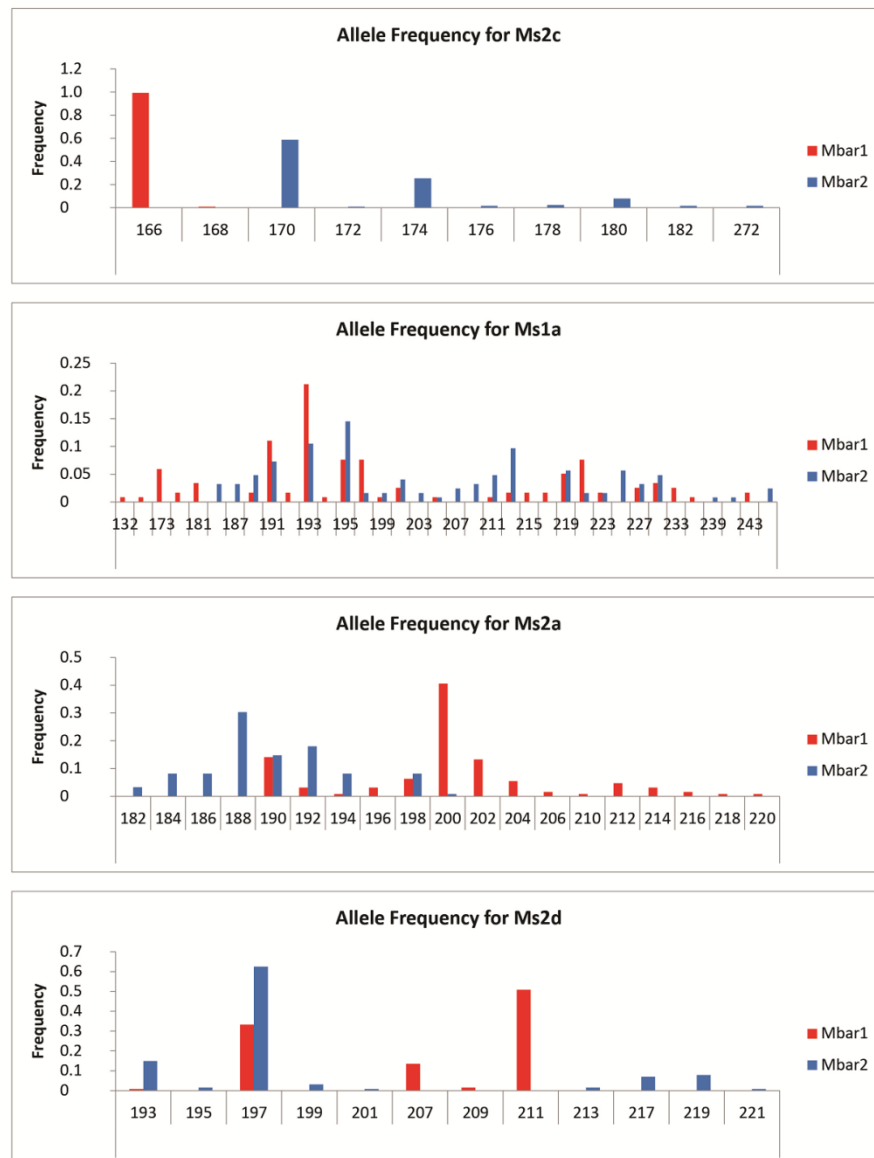


Figure S8.3. Allele frequencies for Mbar1 (red bars) and Mbar2 (blue bars) at the four microsatellite loci surveyed based on the genotypes of 129 reproductive individuals (inferred parents of colonies, and queens and males collected during nuptial flights) with known genotype information at Ms2c

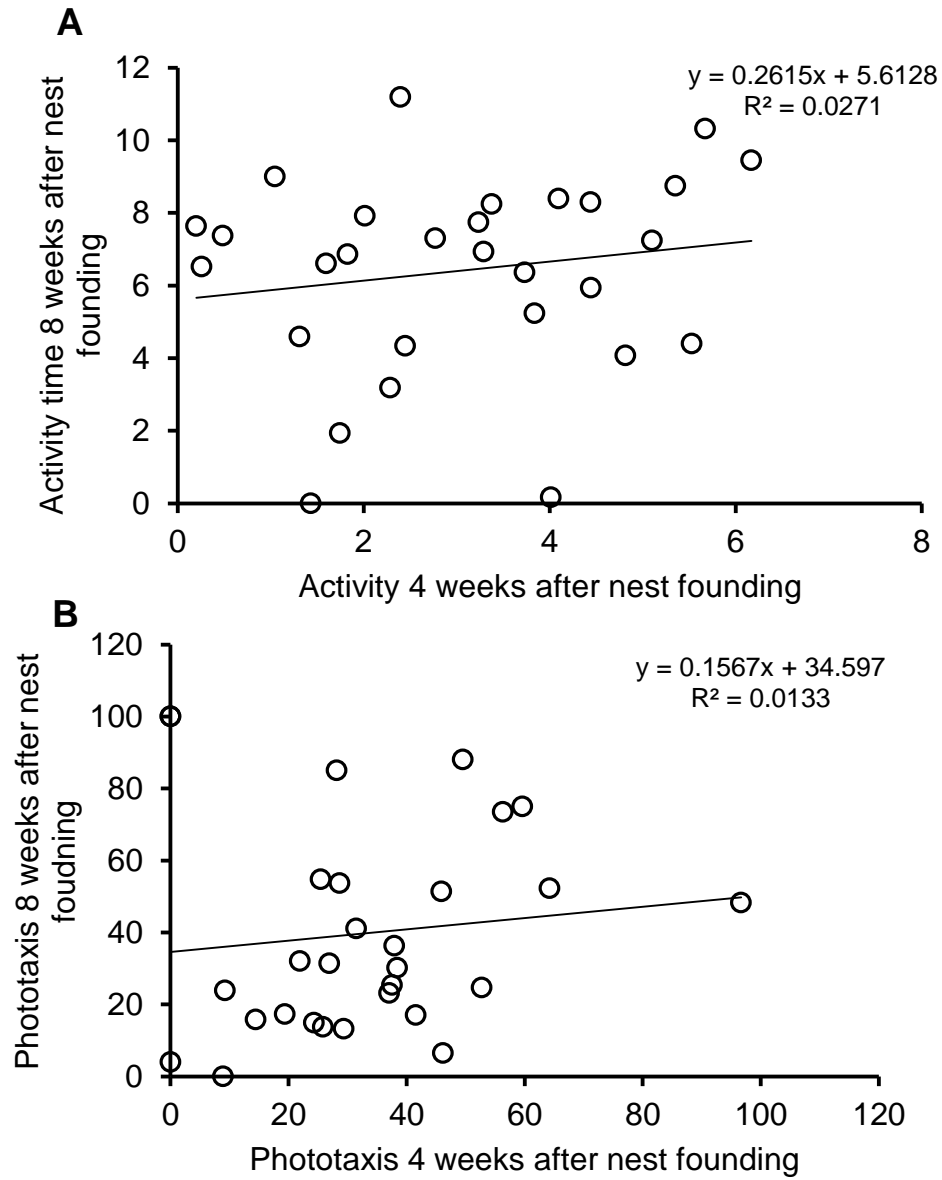


Figure S8.1 Behavioural consistency over two time points (4 and 8 weeks post nest founding), for 28 mated *Lasius flavus* queens for (A) activity level (as measured by AntTrak software) ($T_{27} = 0.86$, $P = 0.39$) and (B) phototaxis (time out of 300s spent in a light environment compared to a dark environment) ($r_s = 0.23$, $P = 0.23$).

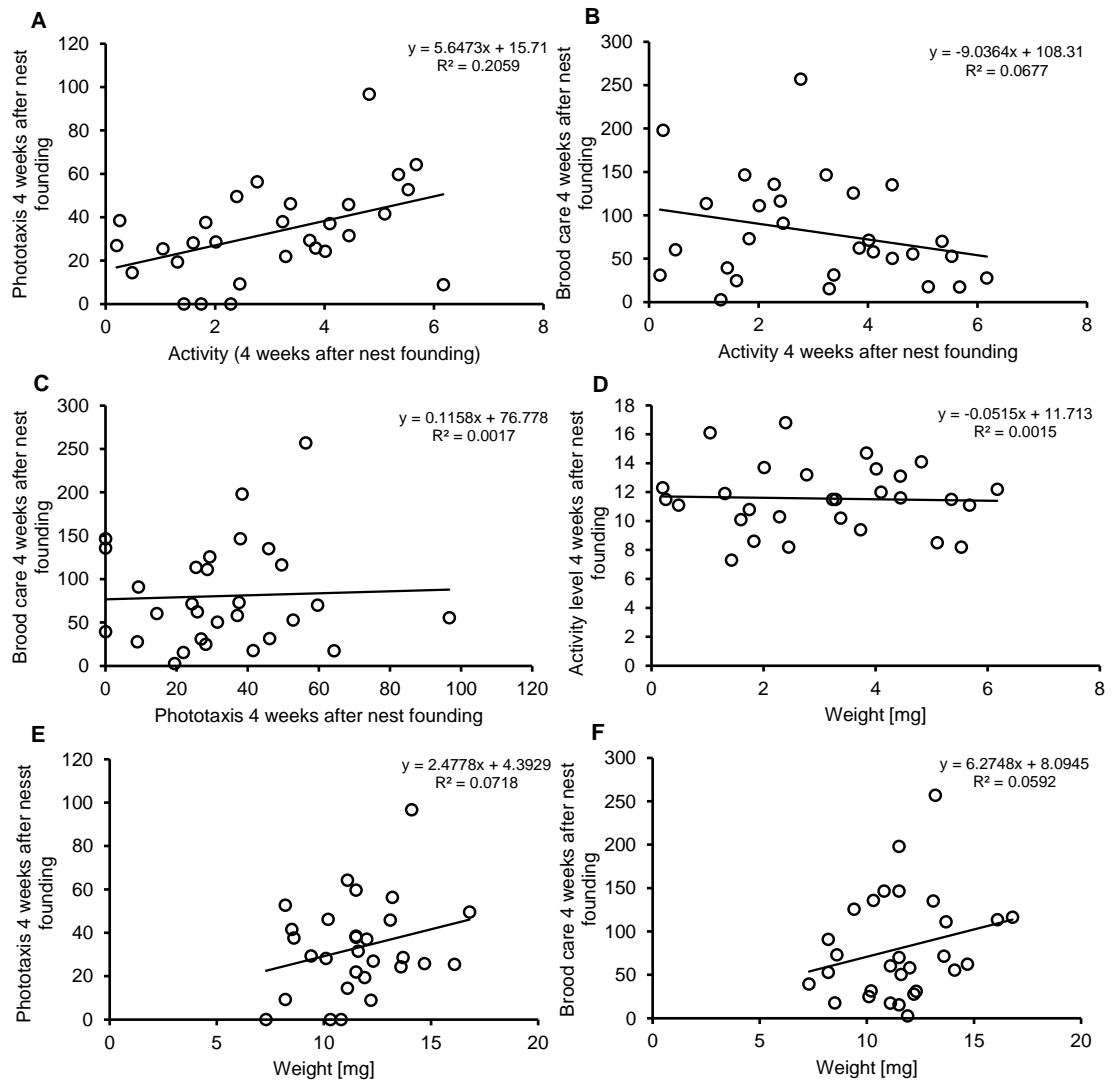


Figure S8.2 Correlations between behavioural traits and queen weights for 28 mated *Lasius flavus* queens 4 weeks post nest founding for (A) phototaxis (time out of 300s spent in a light environment compared to a dark environment) and activity level (as measured by AntTrak software) ($r_s = 0.45$, $P = 0.013$) (B) activity and with brood care tendency (time out of 300s spent tending brood) ($r_s = -0.24$, $P = 0.22$) (C) phototaxis and brood care tendency ($r_s = 0.054$, $P = 0.78$) (D) activity level and queen weight ($T_{27} = -0.20$, $P = 0.84$) (E) phototaxis and weight ($T_{27} = 1.45$, $P = 0.16$) and (F) brood care tendency and weight ($r_s = 0.19$, $P = 0.32$).

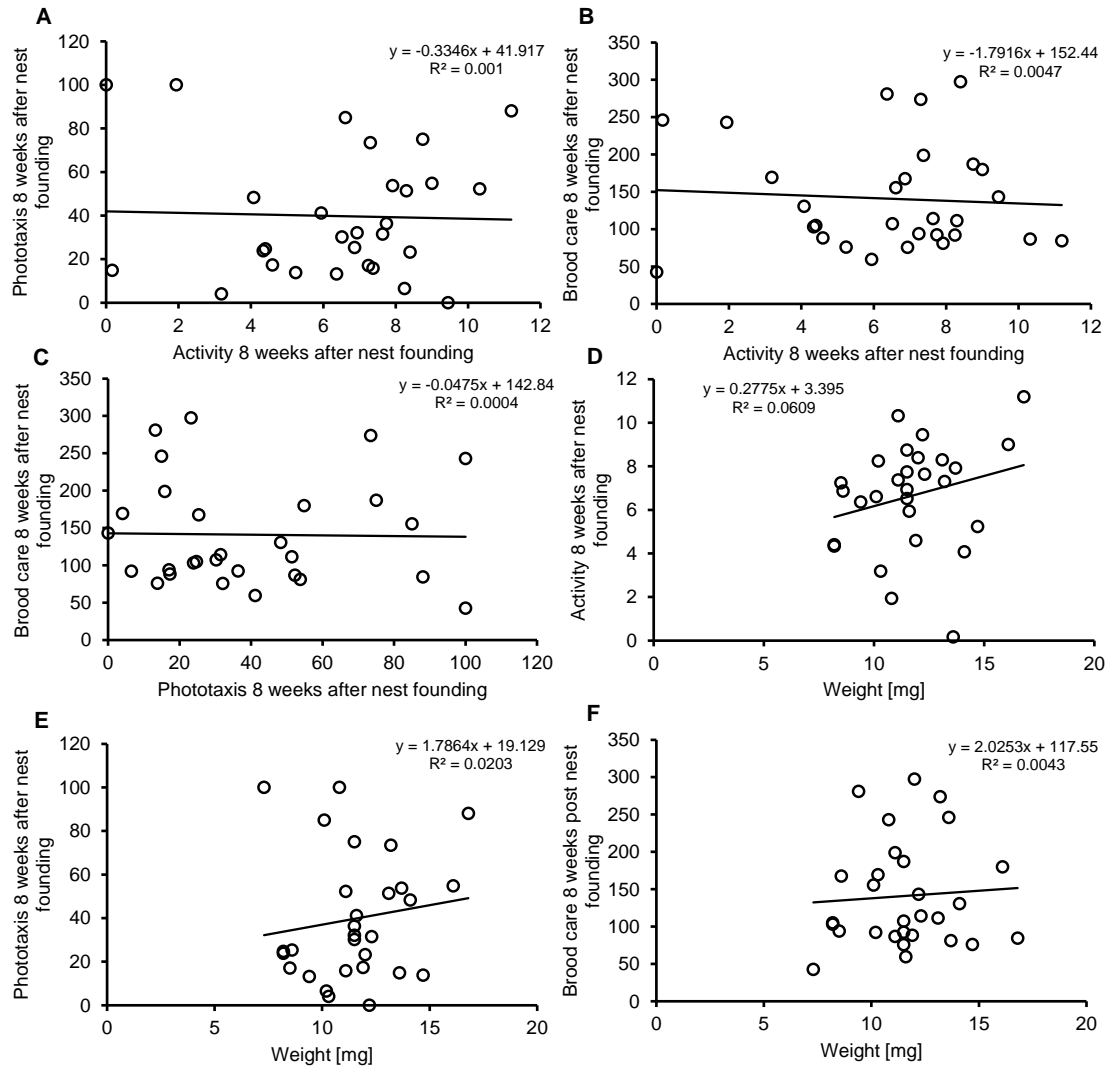


Fig S8.3 Correlations between behavioural traits and queen weights for 28 mated *Lasius flavus* queens 8 weeks post nest founding for (A) phototaxis (time out of 300s spent in a light environment compared to a dark environment) and activity level (as measured by AntTrak software) ($r_s = 0.13$, $P = 0.51$) (B) activity and with brood care tendency (time out of 300s spent tending brood) ($r_s = 0.01$, $P = 0.96$) (C) phototaxis and brood care tendency ($r_s = -0.14$, $P = 0.46$) (D) activity level and queen weight ($T_{27} = 2.04$, $P = 0.051$) (E) phototaxis and weight ($r_s = 0.14$, $P = 0.46$) and (F) brood care tendency and weight ($r_s = 0.02$, $P = 0.91$).

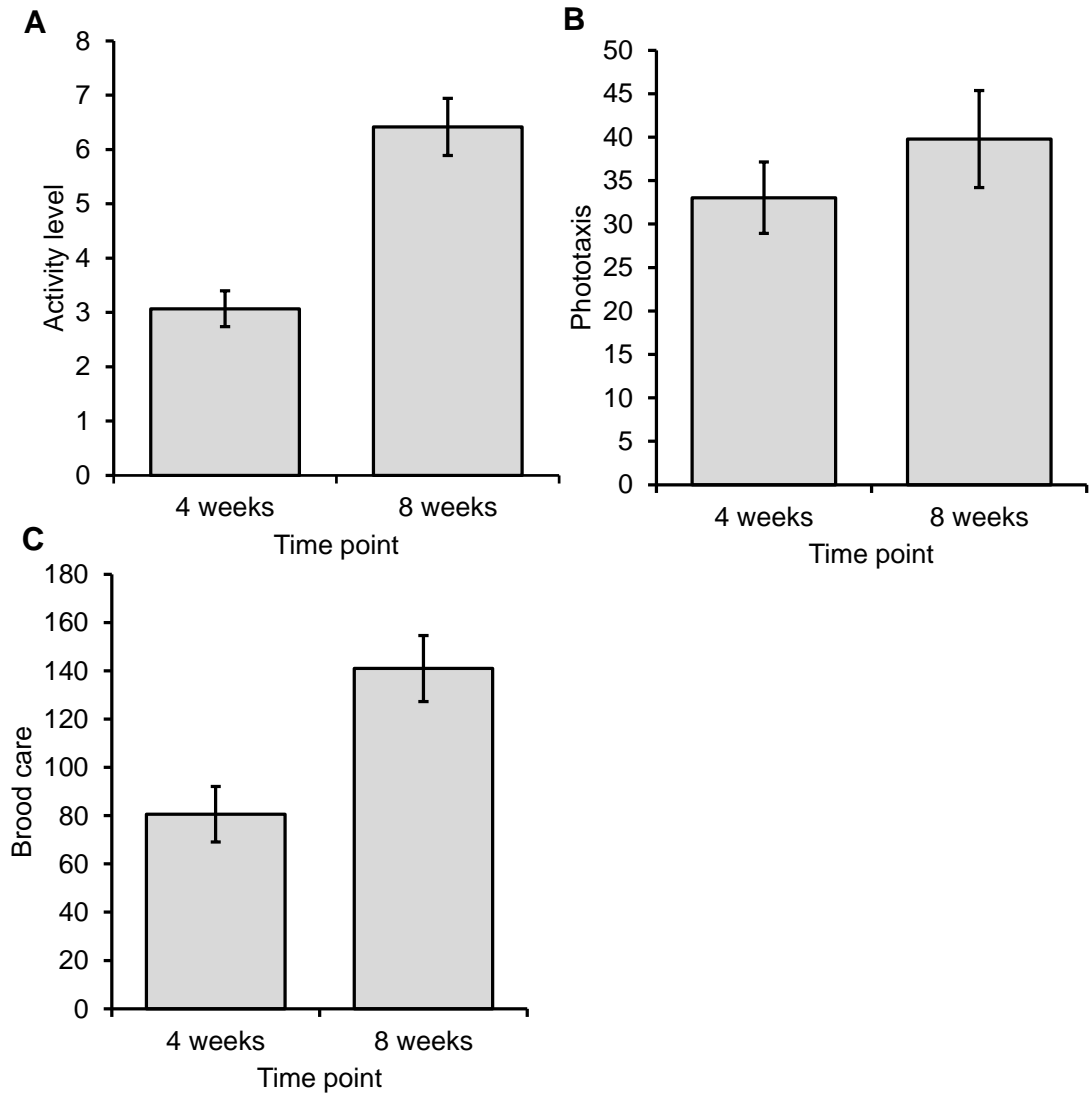


Fig S8.4 Mean \pm S.E of 28 mated *Lasius flavus* queens over two time points (4 weeks post nest founding and 8 weeks post nest founding) for three behavioural traits. Paired t-tests were carried out for (A) activity level (as measured by AntTrak software) ($T_{27} = -6.46$, $P < 0.001$) (B) phototaxis (time out of 300s spent in a light environment compared to a dark environment) ($T_{27} = -0.61$, $P = 0.54$) (C) brood care tendency (time out of 300s spent tending brood) ($T_{27} = -4.5$, $P < 0.001$)

13 Supplementary Tables

Table S3.1 JHa doses used in previous social insect studies including bees, wasps and ants.

Species	Weight	Jha	Jha [$\mu\text{g}/\text{mg}$]	Citation
<i>Polistes dominulus</i>	90mg	5 μg	0.060	1
<i>Polistes dominulus</i>	90mg	25 μg	0.278	2
<i>Acromyrmex octospinosus</i>	13.43mg	3.3 μg	0.223	3
<i>Pogonomyrmex</i> J line	16mg	16 μg	0.625	4
<i>Lasius niger</i> (queen)	27mg	1.1 μg	0.041	5
<i>Apis mellifera</i>	90mg	up to 250 μg	2.770	6
<i>Bombus impatiens</i>	200mg	250 μg	1.250	7
<i>Bombus bimaculatus</i>	200mg	250 μg	1.250	7
<i>Dinoponera quadriceps</i>	350mg	16.5 μg	0.047	Chapter 3

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Table S8.1. Lineage, numbers of worker and gyne (new queens) offspring genotyped, and mating frequency estimates based on offspring genotypes, for 29 Iberian *Messor barbarus* colonies studied. Mating frequency estimates are observed (M_{obs}) and effective (M_{ep}) mating frequencies based on worker (_w) or gyne (_G) offspring. Colonies for which offspring genotypes did not allow parental genotypes to be determined are indicated by nd. The lineage of colonies SOR_c118 and SOR_c141 are unknown.

Colony	Lineage of mother queen	Number of offspring genotyped			# queens	Mating frequency estimates of mother queens			
		workers	gynes	males		M_{obsW}	M_{obsG}	M_{epW}	M_{epG}
ALJ_cA	<i>Mbar1</i>	89			1	3		2.22	
ALJ_cB	<i>Mbar2</i>	92			1	2		1.89	
ALC_c1	<i>Mbar2</i>	4			nd	nd		nd	
BAU_c1	<i>Mbar1</i>	4		4	1	1		1.00	
BAU_cA	<i>Mbar2</i>	4		4	1	2		2.25	
BAU_cB	<i>Mbar1</i>	4		1	1	nd		nd	
CAC_c1	<i>Mbar2</i>	6	3	3	1	nd	2	nd	1.80
MOJ_c1	<i>Mbar2</i>	4			nd	nd		nd	
POZ_c1	<i>Mbar1</i>	8			1	3		3.61	
SOR_c1	<i>Mbar1</i>	6	8	1	1	nd	2	nd	1.32
SOR_c2	<i>Mbar2</i>	5	2	4	1	2	1	2.25	1.00
SOR_c3	<i>Mbar2</i>	5	6	4	1	2	2	2.17	1.99
SOR_c4	<i>Mbar1</i>	5	10		1	1	1	1.00	1.00
SOR_c5	<i>Mbar2</i>	6	3	3	1	3	2	2.27	1.80
SOR_c6	<i>Mbar1</i>	5		6	1	2		2.17	
SOR_c7	<i>Mbar1</i>	5		6	1	2		2.17	
SOR_c8	<i>Mbar2</i>	9	1	5	1	4	nd	3.69	nd
SOR_c9	<i>Mbar1</i>	9	2		1	1	nd	1.00	nd
SOR_c10	<i>Mbar2</i>	9	9	4	1	3	1	3.36	1.00

SOR_c11	<i>Mbar2</i>	7	3	7	1	3	1	2.74	1.00
SOR_c104	<i>Mbar2</i>	13			1	4		2.82	
SOR_c117	<i>Mbar2</i>	13			nd	nd		nd	
SOR_c118	nd	14			1	2		2.09	
SOR_c121	<i>Mbar1</i>	15			1	2		1.89	
SOR_c126	<i>Mbar1</i>	14			1	3		3.17	
SOR_c129	<i>Mbar1</i>	11			nd	nd		nd	
SOR_c131	<i>Mbar2</i>	13			1	4		4.16	
SOR_c132	<i>Mbar1</i>	13			1	2		1.61	
SOR_c137	<i>Mbar1</i>	12			1	3		2.58	
SOR_c140	<i>Mbar2</i>	11			1	2		2.15	
SOR_c141	nd	13			nd	nd		nd	
SOR_c145	<i>Mbar1</i>	16			1	2		1.14	
SOR_c147	<i>Mbar1</i>	9			1	2		1.95	
SOR_c148	<i>Mbar1</i>	10			nd	nd		nd	
SOR_c151	<i>Mbar1</i>	15			1	3		1.56	
SOR_c155	<i>Mbar1</i>	13			1	2		1.61	

Table S8.1 Statistical results from a PCA analysis assessing combinations of three behavioural traits and queen weight at two time periods in colony foundation (4 and 8 weeks post nest founding) for 28 mated *Lasius flavus* queens. Here we present those axes created by the PCA but where the eigenvalue is less than 1 (meaning these axes were not used for subsequent analyses).

PCA during the first period	Factor loadings		PCA during the second period	Factor loadings
	PC3	PC4		PC4
Eigenvalue	0.691	0.432	Eigenvalue	0.574
% variation	17.272	10.807	% variation	14.351
broodcare	0.68	0.242	broodcare	0.221
activity	0.206	0.659	activity	0.64
phototaxis	0.193	-0.665	phototaxis	0.284
weight	-0.677	0.255	weight	-0.679